CHEMICAL CHARACTERIZATION OF GANODERMA LUCIDUM AND ITS ASSOCIATED BIOACTIVITIES IN CULTURED HUMAN CARCINOMA CELLS

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NATIONAL UNIVERSITY OF SINGAPORE

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DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously

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LIST OF ABBREVIATIONS

7-AAD: 7-Amino-actinomycin D

ANOVA: analysis of variance

AP-1: activator protein 1

Apaf1: apoptotic protease activating factor 1

APCI: atmospheric pressure chemical ionization

Bax: Bcl-2–associated X protein

BBD: Box–Behnken factorial design

Bcl-2: B-cell lymphoma 2

BSA: bovine serum albumin

Cdk: cyclin-dependent kinase

C/EBP: CCAAT/enhancer-binding proteins

DC-STAMP: dendritic cell−specific transmembrane protein

DEPT: distortionless enhancement of polarisation transfer

DHA: docosahexaenoic acid

DMEM: Dulbecco's modified Eagle's medium

DMSO: dimethyl sulfoxide

ESI-MS: electrospray ionization mass spectrometry

ERK: extracellular signal-regulated kinases

FAS: fatty acid synthase

FITC: fluorescein isothiocyanate

GPDH: glycerol-3-phosphate dehydrogenase

HIF: hypoxia-inducible factor

HPLC: high performance liquid chromatography

IL-6: interleukin-6

JNK: c-Jun N-terminal kinases

LPS: lipopolysaccharide

MAPK: mitogen-activated protein kinases

MMP: matrix metalloproteinase

MS: mass spectrometry

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide

NFATc1: nuclear factor of activated T cells c1

NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells

NMR: nuclear magnetic resonance

ONPG: o-nitrophenyl-β-D-galactopyranoside

PARP: poly ADP-ribose polymerase

PBS: phosphate buffered saline

PDA: Photodiode Array

PDGF: platelet-derived growth factor

PI: propidium iodide

PKC: protein kinase C

PMA: phorbol-12-myristate-13-acetate

PPAR: peroxisome proliferator-activated receptor

RSM: response surface methodology

SFE: supercritical fluid extraction

SRE: serum response element

SR-VAD-FMK: sulforhodamine - valyl - alanyl - fluoromethyl – ketone

tBid: truncated BH_3 interacting domain death agonist

Tcf/Lef: T-cell Factor / Lymphocyte Enhancer binding Factor

TGFβ: transforming growth factor beta

TLC: thin layer chromatography

TNF-α: tumor necrosis factor-α

TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling

uPA: urokinase-type plasminogen activator

SUMMARY

The medicinal mushroom *Ganoderma lucidum* has been reported to be effective herbal remedy in traditional medicine for its health promoting properties. Studies regarding the bioactivity of *Ganoderma lucidum* have thrived across diverse research areas including cancer, diabetes, immune system, hyperglycemia and anti-viral [1-6]. The major chemical components of *Ganoderma lucidum* that are believed to be responsible for these effects include polysaccharides and triterpenoids. Compared to polysaccharides, *Ganoderma lucidum* triterpenoids are suggested to have more direct effects in cancer prevention. Both the triterpenoid extracts and individual triterpenoids including their derivatives have been reported to inhibit cell proliferation and induce cell deaths in a number of carcinoma cell models [7]. The exact mechanisms for their chemo-preventative properties remain unclear, but it is likely due to the diverse chemical structures of *Ganoderma lucidum* triterpenoids and their derivatives.

Though there have been extensive studies on *Ganoderma lucidum* triterpenoids, literature is sparse in evaluating the associated bioactivity of other components in *Ganoderma lucidum* such as lipids, proteins and nucleosides. In recent years, increasing interests have been addressed in chemical and bioactive characterization of the lipid constituents in *Ganoderma lucidum.* Lipids mixture and long chain fatty acids have been found to induce apoptosis in human leukemia cells [8;9]. While understanding of the compositions in these extracts and bioactivity in other carcinoma cells is still limited.

Thus, this thesis is focusing on investigating the specific bioactivities of *Ganoderma lucidum* lipids and triterpenoids and their derivatives in three cultured human carcinoma cells. The overall results indicated that *Ganoderma lucidum*

triterpenoids and their derivatives were able to inhibit cell proliferations in Caco-2 human colon carcinoma cells, HeLa human cervical carcinoma cells and Hep G2 human hepatic carcinoma cells and induce apoptotic cell death in Caco-2 and HeLa cells. Triterpenoids with functional groups at C-23 position, less saturation in the side chain and higher polarity were suggested to have impaired cytotoxicity in these cell lines. The *Ganoderma lucidum* triterpenoids induced-apoptosis was associated with caspase activation, membrane protein externalization and cytochrome c release, possibly through transcription activation of p53 and E2F and down regulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), Myc and hypoxia-inducible factor (HIF). On the other hand, the *Ganoderma lucidum* lipids were shown to induce differentiation in Caco-2 cells with accelerated alkaline phosphatase and lactase activity and this action was possibly mediated through activation of c-Jun N-terminal kinases (JNK) and extracellular signal-regulated kinases (ERK) signaling and involved an indirect activation of protein kinase C (PKC). In addition, reliable and rapid methods for separating and characterizing *Ganoderma lucidum* lipids and triterpenoids and their derivatives have been developed. Extraction conditions including extraction time, temperatures and solvents were evaluated and optimized to obtain maximum yields of *Ganoderma lucidum* triterpenoids.

The evidence presented in this thesis supports the hypothesis that *Ganoderma lucidum* lipids, triterpenoids, and their derivatives exhibit strong bioactivities in cultured human carcinoma cells. Detailed studies on the possible direct targets in the regulation signaling would be required for complete elucidation of the specific underlying mechanisms. Additionally, future studies

may need to be developed for confirming their pharmacological effects in vivo as the potential chemotherapeutic agents against cancers.

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CHAPTER 1 INTRODUCTION

1.1 Overview of Bioactive Components from *Ganoderma lucidum*

Ganoderma lucidum, also known as Lingzhi in Chinese, Reshi in Japanese and Youngzhi in Korean, is a medicinal herb traditionally used in ancient Asia to promote health and cure various diseases and is first recognized in the west in the 18th century [10]. Modern research has revealed that *Ganoderma lucidum* possesses multiple pharmacological activities, including anti-inflammatory, anti-tumorigenic, anti-herpetic, anti-virus, anti-ulcerogenic, anti-angiogenic, anti-diabetic, hypoglycemic activity and osteoclastogenesis-regulated effects [3;6;11-17]. *Ganoderma lucidum* has been reported to consists of a variety of chemical components with bioactive properties, which include polysaccharides, triterpenoids, steroids, fatty acids, proteins, peptides, alkaloids and nucleosides [18]. Among these ingredients, polysaccharides and triterpenoids are the two most extensively studied components. Structure-activity relationship has been indicated in the anti-angiogenesis effects and anti-oxidant capacity in *Ganoderma lucidum* polysaccharides [19;20]. Yet little is known of the correlation between the structures of *Ganoderma lucidum* triterpenoids and their associated bioactivities. The potential bioactive lipid components present in *Ganoderma lucidum* has also been garnering recent interest [8;21].

1.2 Objective of Thesis

Overall Aim

The overall objective of this thesis is to develop and optimize methods for extraction, isolation, characterization of *Ganoderma lucidum* triterpenoids and lipids and to assess their respective bioactive properties in three cultured human carcinoma cell models.

Specific Objectives

1. To develop crude *Ganoderma lucidum* extracts and generate fractions that contain enriched triterpenoids (Chapter 3)

2. To assess bioactive response of Caco-2 cells to two *Ganoderma lucidum* triterpenoids enriched fractions (Chapter 3)

3. To characterize and quantify a *Ganoderma lucidum* lipid enriched fraction (Chapter 4)

4. To investigate the induction of differentiation on Caco-2 cells resulted from *Ganoderma lucidum* lipid enriched fraction (Chapter 4)

5. To model effects of extraction conditions on triterpenoid profiles and develop optimized extraction conditions for certain triterpenoids (Chapter 5)

6. To develop methods for isolation and identification of individual triterpenoids and their derivatives (Chapter 5 and 6)

7. To measure the corresponding anti-proliferative effects of isolated individual triterpenoids and their derivatives on Caco-2, HeLa and Hep G2 cells (Chapter 5 and 6)

8. To quantify the levels of apoptosis induced by triterpenoids and their derivatives and further elucidate possible cellular signaling pathways (Chapter 7)

CHAPTER 2 LITERATURE REVIEW

Preface

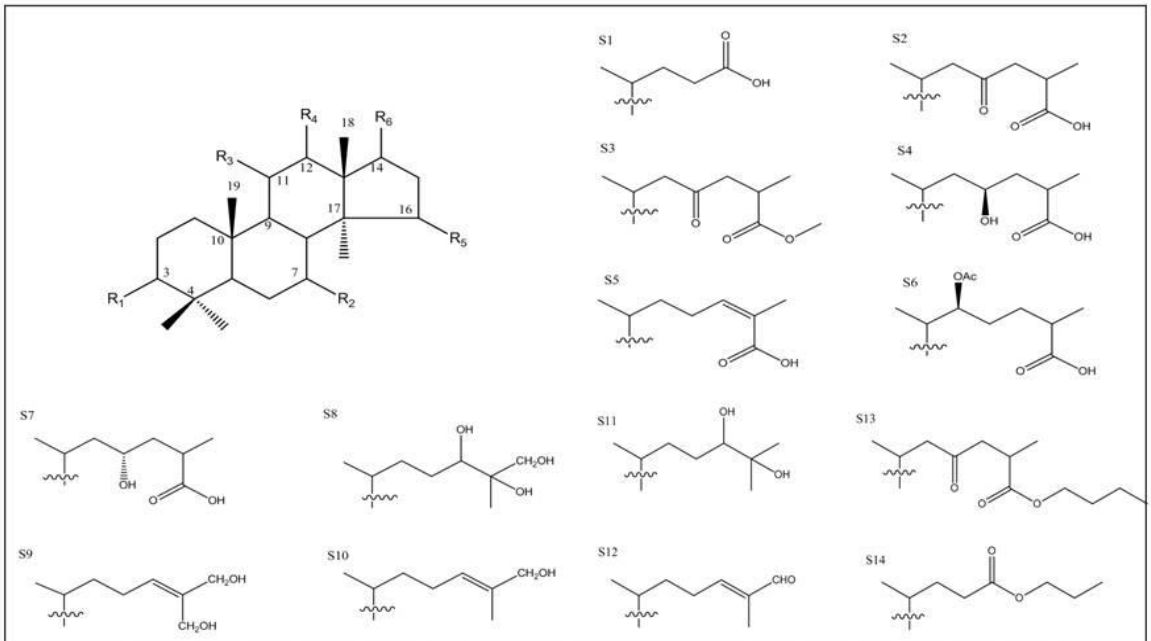
Selected portion of Chapter 2 have been published in the following publication

Ruan, W.; Popovich, D. G. Evidence of bioactivity from *Ganoderma lucidum* triterpenoids in cultured cell models. In *Saponins: Properties, Applications and Health Benefits,* Nova Science Publishers. **2011**, 145-156.

2.1 Overview of Triterpenoids from *Ganoderma lucidum*

2.1.1 Extraction, Isolation and Identification of *Ganoderma lucidum* Triterpenoids

Triterpenoids derived from *Ganoderma lucidum* are oxygenated tetracyclic triterpenoids. The majority of these triterpenoids are ganoderic acids (C30) and lucidenic acid (C27). In addition to the two major triterpenoids, ganolucidic acids, ganoderenic acids, ganoderiols, ganodermadiol, ganodermatriol, ganodermanondiol, ganodermanontriol, lucidumols, ganoderic aldehydes and lucialdehydes are also found in *Ganoderma lucidum*. New triterpenoids are continuously discovered by different research groups. For example, 7-O-ethyl ganoderic acid O was newly found from fermented mycelia of *Ganoderma lucidum* by silica gel chromatography [22]. A novel ganoderic acid, ganoderic acid FWI was found in a methanolic extract [23]. Additionally, new triterpenoid derivatives such as ganofuran B, butyl ganoderate and butyl lucidenate were discovered [2;24]. Nevertheless, many more triterpenoids from *Ganoderma lucidum* remain undiscovered and worthwhile to explore. Chemical structures of representative triterpenoids identified from *Ganoderma lucidum* are shown in **Figure 1**.



Compound	R1	R2	R3	R4	R5	R6	D.B.#	Compound	R1	R2	R3	R4	R5	R6	D.B.#
Lucidenic acid A	O	βOH	O	H	O	S1	8,9	Ganodermanontriol	O	H	H	H	H	S8, 24-βOH	7,8;9,11
Lucidenic acid D2	O	O	O	βOAc	O	S1	8,9	Ganoderiol B	O	H	H	H	OH	S9	7,8;9,11
Ganoderic acid A	O	βOH	O	H	OH	S2	8,9	Ganoderiol D	O	O	H	H	H	S8, 24-αOH	8,9
Ganoderic acid C	βOH	βOH	O	H	OH	S2		Ganodermadiol	βOH	H	H	H	H	S10	7,8;9,11
Ganoderic acid B methyl ester	βOH	βOH	O	H	O	S3	8,9	Lucidumol A	O	O	H	H	H	S11, 24-βOH	8,9
Ganoderic acid DM	O	O	H	H	H	S5	8,9	Ganodermanondiol	O	H	H	H	H	S11, 24-αOH	7,8;9,11
Ganoderic acid F	O	O	O	βOAc	O	S2	8,9	Ganoderic aldehyde TR	O	H	H	H	OH	S12	7,8;9,11
Ganoderic acid R	βOAc	H	H	H	OAc	S5	7,8;9,11	Lucialdehyde B	O	O	H	H	H	S12	8,9
Ganoderic acid LM2	O	βOH	O	H	O	S4	8,9;24,25	7-O-ethyl ganoderic acid O*	OAc	OCH_2CH_3	H	H	OAc	S6	8,9;24,25
Ganoderic acid T	OAc	H	H	H	OAc	S6	7,8;9,11	Butyl ganoderate A*	O	βOH	O	H	OH	S13	8,9
Ganoderic acidε	βOH	βOH	O	H	O	S7	8,9;24,25	Butyl lucidenate N*	βOH	βOH	O	H	O	S14	8,9
Ganolucidic acid D	O	H	O	H	OH	S4	8,9								
Ganoderenic acid A	O	βOH	O	H	OH	S2	8,9;20,22								

Figure 1. Chemical structures of triterpenoids from *Ganoderma lucidum* # refers to double bonds, * represents newly discovered triterpenoids in recent years by different research groups.

Due to the complex profile of triterpenoids from *Ganoderma lucidum*, extraction and isolation of triterpenoids from *Ganoderma lucidum* usually involves multiple steps of

separation and purification. Organic solvents such as methanol, ethanol and chloroform are commonly used as extraction solvents and various methods of isolation including repeated silica gel column chromatography, C18 cartridge, preparative high performance liquid chromatography (HPLC), capillary zone electrophoresis and supercritical fluid extraction (SFE), has been employed to obtain purified triterpenoid extracts or individual triterpenoids [25-29]. However, effects of extraction conditions on recovery yields of certain triterpenoids remain unclear.

Identification of *Ganoderma lucidum* triterpenoids using thin layer chromatography (TLC) has been reported as an economic and simple approach while HPLC coupled with UV detector are employed prevalently [29-31]. However, since triterpenoids are not the exclusive components absorbed in the detective UV wavelengths and standards for many triterpenoids are not commercially available; LC-MS is generally required for accurate identification of triterpenoids from *Ganoderma lucidum*. Electron spray ionization (ESI) and atmospheric pressure chemical ionization (APCI) have been used in assisting confirmation of the structures of triterpenoids with same molecule weight by examination of the fragmentation patterns including the cleavage of A, B, C, D ring, loss of methyl radical and fragmentation of side chain [32;33]. Yet there are few reports of such fragmentation patterns on triterpenoid derivatives and exact identification may need combining analysis of ESI and nuclear magnetic resonance (NMR).

2.1.2 Bioactivities of *Ganoderma lucidum* Triterpenoids in Cell Culture

2.1.2.1 Anti-Inflammatory, Adipogenesis and Osteoclast Differentiation Effect in Cultured Cells

Triterpenoids from *Ganoderma lucidum* have been reported to exhibit diverse bioactivity in variety of cell culture models. Extracts of triterpenoids from *Ganoderma*

lucidum possessed anti-inflammatory effects on lipopolysaccharide (LPS)-stimulated macrophages by suppressing secretion of potentially harmful cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) [34].

Butyl ganoderate A and butyl lucidenate N isolated from *Ganoderma lucidum* were demonstrated to inhibit adipogenesis in 3T3-L1 cells by down-regulation of the adipogenic transcription factor sterol regulatory element-binding protein -1c and its target genes, such as fatty acid synthase (FAS) and acetyl-CoA carboxylase [35]. Another triterpenoid T-butyl lucidenate B was also shown to exert adipogenesis inhibitory effect by reduction of triglyceride accumulation and suppression of glycerol-3-phosphate dehydrogenase (GPDH) activity and gene expressions of peroxisome proliferator-activated receptor (PPAR) γ, CCAAT/enhancer-binding proteins (C/EBP) α, and sterol regulatory element-binding protein -1c [36]. Recently, a *Ganoderma lucidum* extract containing approximately 10% of ganoderic acid A was reported to promote adipocyte differentiation and adiponectin production by activating PPARγ and PPARα [37]. Production of adiponectin is associated with an improved metabolic potential, which is the adaptive response with increasing oxidative potential and greater ability to use fat as a fuel.

Triterpenoids from *Ganoderma lucidum* could be beneficial in osteoclastogenesis therapeutics. Inhibition of osteoclast differentiation helps in preventing excessive osteoclastic bone resorption, which is believed to be responsible for osteopenic diseases such as osteoporosis and rheumatoid arthritis. Ganoderic acid DM and ganoderic acid F were found to exhibit cytotoxicities and inhibit osteoclast differentiation in RAW264 mouse leukemic monocyte-macrophage cells. These effects were mediated by suppression of c-Fos and the nuclear factor of activated T cells c1 (NFATc1) which subsequently suppressed expression of dendritic cell-specific transmembrane protein

(DC-STAMP) and reduced osteoclast fusion [38]. Additionally, a structure-activity relationship was believed to exist in the inhibitory activity [12].

2.1.2.2 Chemoprevention

The most extensively studied of *Ganoderma lucidum* triterpenoids bioactivity is their anti-tumor or chemo-preventative effects. Both triterpenoid extracts from *Ganoderma lucidum* and individual isolated triterpenoids have been reported to inhibit cell growth and cause cell deaths in various carcinoma cells. Preliminary research mainly focused on the cytotoxic properties of triterpenoids from *Ganoderma lucidum* on tumor cells. For instance, ethanol extracts from *Ganoderma lucidum* have been studied using human cervical carcinoma HeLa cells [39]. Additionally, individual triterpenoids including lucidenic acid N, lucidenic acid A, and ganoderic acid E have been showed significant cytotoxicity in Hep-G2 and murine P-388 lymphocytic leukemia cells [40]. Recently, a possible mechanism of the anti-tumor properties has been partly elucidated. Molecular mechanisms involved include multiple programmed cell death such as apoptosis, autophagy and senescence and reduce or prevent cellular metastasis. The chemo-preventative properties from *Ganoderma lucidum* triterpenoids are summarized in **Table 1**.

Table 1. Summary of the type of cell death in specific cell lines using triterpenoids from *Ganoderma lucidum.*

Components	Responsive cell lines	Type of cell death	Molecular mechanisms involved	Reference
Methanol extracts	transplantable B16 murine melanoma cells, mouse fibrosarcoma L929 cells ,rat astrocytoma C6 cells	Apoptosis and necrosis	Up regulation of 1. p53 2. caspase 3 Down regulation of 1. Bcl-2	[41]
Methanol extracts	interleukin 3-dependent lymphoma DA-1cells	Apoptosis	Down regulation of 1. NFkB-p65 2. Inactive caspase 3	[42]
Methanol	NB4 human leukemia cells.	Apoptosis	Down regulation of	[43]

extract with possible triterpenoids			1. p53 2. Akt 3. Erk 4. Bcl2/Bax ratio.	
Triterpenoid-rich ethanol extract	platelet-derived growth factor (PDGF-BB)-activated rat hepatic stellate cells (HSC-T6)	Apoptosis	Up regulation of 1. JNK Down regulation of 1. cyclins D1 and D2 2. PDGFβR 3. Akt 4. α-SMA.	[44]
Ethanol extracts of triterpenoids	human hepatoma Hep 3B and HepG2 cells, breast cancer cells (MCF-7 and MDA-MB-231) and myeloid leukemia cells (HL-60)	Apoptosis	Not Detected	[45;46]
Ethanol extract	AGS human gastric carcinoma cells	Apoptosis	Up regulation of 1. death receptor 5 2. TRAIL 3. caspase 3,8 and 9 Down regualtion of 1. Akt 2. Bcl-2 3. Bid 4. XIAP 5. survivin Degradation of poly (ADP-ribose) polymerase	[47]
Triterpene-enriched extract	SW620 human colorectal carcinoma cells	Apoptosis	Up regulation of 1. p53 2. caspase 3 Down reguation of 1. Bcl-2/Bax ratio	[48]
Ganoderic acid Me	HCT-116 human colon carcinoma cells	Apoptosis	Up regulation of 3. p53 4. caspase 3 Down reguation of 1. Bcl-2/Bax ratio 2. Mitochondria membrane potential Release of cytochrome c	[49]
Ganoderic acid Mf and ganoderic acid S	human cervical carcinoma HeLa cells	Apoptosis	Down regulation of 1. Bcl-2/Bax ratio 2. caspase 3and 9 3. mitochondria membrane potential	[50]

			Release of cytochrome c	
Triterpenoids from *Ganoderma lucidum*	HT-29 human colon cancer cells	Autophagy	Up regulation of 1. Beclin-1 2. LC-3 Down regulation of 1. p38 MAPK Formation of autophagic vacuoles	[51]
Ganoderiol F	HepG2 cells.	Senescence	Up regulation of 1. EKR 2. p16 Inhibition of 1. DNA synthesis 2. topoisomerases activity	[52]
Ganoderic acid A and ganoderic acid H	MDA-MB-231 breast cancer cells	Antimetastasis	Down regulation of 1. AP-1 2. NF-κB 3. Cdk4 4. secretion of uPA	[53]
Ganoderic acid T	95-D human highly metastatic lung tumor cells	Antimetastasis	Down regulation of 1. MMP-2 and 9 2. NF-κB.	[54]
Lucidenic acids A, B, C and N	phorbol-12-myristate-13-acetate (PMA)-induced HepG2 cells	Antimetastasis	Down regulation of 1. MMP-9 2. ERK1/2 3. AP-1 4. NF-kB	[55]

2.1.2.2.1 Programmed Cell Death

Apoptosis

One of the most commonly studied bioactive properties of natural products is the ability to inhibit apoptosis in cancer cell lines. Methanol extracts containing ganoderic acids and triterpenoid derivatives were found to induce apoptosis and necrosis in transplantable B16 murine melanoma cells, mouse fibrosarcoma L929 cells and rat astrocytoma C6 cells. The apoptotic cell death involved was caspase-dependent and mediated by up-regulation of tumor suppressor protein p53 and suppression of

anti-apoptotic protein B-cell lymphoma -2 (Bcl-2) expression [41]. Another methanol extract possibly containing triterpenoids was also indicated to cause apoptosis in interleukin 3-dependent lymphoma cells (DA-1) with reduction of expression of NFkB-p65 factor and inactivated caspase-3 [42]. Additionally, induction of apoptosis in NB4 human leukemia cells was found with treatment of a similar methanol extract possibly containing triterpenoids. The corresponding activity involved reductions of p53, Akt, Erk level as well as the Bcl2/ Bcl-2-associated X protein (Bax) ratio [43].

Triterpenoid enriched extract by ethanol extraction was shown to induce apoptosis in platelet-derived growth factor (PDGF)-activated rat hepatic stellate cells (HSC-T6) and it was mediated by down-regulation of cyclins D1 and D2, PDGFβR and Akt phosphorylation, up-regulation of JNK phosphorylation, and expression of α-SMA [56]. Ethanol extracts of triterpenoids from *Ganoderma lucidum* also induced apoptosis in human hepatocarcinoma Hep3B and HepG2 cells [45;46]. However, the underlying molecular mechanisms were not elucidated. In addition to hepatocarcinoma cells, ethanol extracts exhibited anti-proliferative activities on human breast cancer cells (MCF-7 and MDA-MB-231), myeloid leukemia cells (HL-60) and AGS human gastric carcinoma cells through induction of apoptosis [45;47]. Induction of apoptosis in AGS human gastric carcinoma cells was associated with two distinct pathways including extrinsic plasma membrane death receptor-mediated pathway and intrinsic mitochondria-mediated pathway which corresponded to inactivation of Akt [47]. More recently, a triterpene-enriched extract was found to induce apoptosis in SW620 human colorectal carcinoma cells by activating caspase-3, up-regulating p53 and down-regulating Bcl2/Bax ratio [48].

Individual triterpenoid, ganoderic acid Me was reported to induce apoptosis in HCT-116 human colon carcinoma cells through mitochondrial mediated pathway.

Increased expression of p53 and reduced Bcl-2/Bax ratio were reported [49]. The induction of apoptosis was also coupled with reduced mitochondria trans-membrane potential, released cytochrome c from mitochondria into the cytosol and increased caspase-3 activity. Following a similar mechanism induced by ganoderic acid Me in HCT-116cells, a pair of position isomer, ganoderic acid Mf and ganoderic acid S were able to trigger apoptotic cell death in HeLa cells. The actions involved decreased Bcl-2/Bax ratio, activated caspase-3 and caspase-9, decreased mitochondria membrane potential and release of cytochrome c [50].

Autophagy and Senescence

Another type of programmed cell death is termed autophagy and it may be responsible for part of the cytotoxic properties and mechanisms attribute to the chemo-prevention properties of *Ganoderma lucidum* triterpenoids. Triterpenoids from *Ganoderm lucidum* induced autophagic cell death in HT-29 human colon cancer cells, which was associated with formation of autophagic vacuoles, up-regulation expression of Beclin-1 and LC-3 protein. These effects were suggested to be mediated by inhibition of p38 mitogen-activated protein kinase (MAPK) [57].

When programmed cell death is blocked in cells, irreversible cell growth arrest, termed senescence has also been reported activated by *Ganoderma lucidum*. Ganoderiol F has been reported to induce senescence in HepG2 cells. The inhibition of DNA synthesis was presumably caused by the inhibition of topoisomerases activity and activation of EKR and up-regulation of cyclin-dependent kinase inhibitor p16 might contribute to the premature senescence induction [52].

2.1.2.2.2 Anti-Invasion and Anti-Metastasis

In addition to suppressing the growth of existing tumors, preventing invasion and metastasis are also important mechanisms for either treatment. Ganoderic acid A and ganoderic acid H were shown to exhibit anti-invasive effects on MDA-MB-231 cells [58]. These effects were mediated by down-regulation of transcription factors activator protein -1 (AP-1) and NF-κB, subsequently suppressed expression of cyclin-dependent kinase (Cdk) 4 and secretion of urokinase-type plasminogen activator (uPA), respectively. Structure-activity relationship was also suggested. Ganoderic acid T was also demonstrated to effectively inhibit tumor metastasis in 95-D human highly metastatic lung tumor cells via blocking expression of matrix metalloproteinase (MMP) -2 and MMP-9 which could be mediated by inactivation of NF-κB [54]. Lucidenic acids A, B, C and N were found to possess anti-invasive activity on phorbol-12-myristate-13-acetate (PMA)-induced Hep G2 cells by down regulating MMP-9 activity. Inhibition of ERK1/2 phosphorylation and reduction of DNA-binding activities of AP-1 and NF-kB were reported to be involved in this process [59].

Triterpenoids from *Ganoderma lucidum* have been reported to possess diverse bioactive properties and perhaps the most convincing evidence for bioactivity is the chemo-preventative properties. These triterpenoids may induce activity mediated though activation of multiple programmed cell death pathways. However, elucidating a structure-activity relationship on specific cancer cell lines using individual isolated triterpenoids is still limited.

2.2 Overview of Lipids from *Ganoderma lucidum*

2.2.1 Chemical Characterization of Lipids from *Ganoderma lucidum*

Unlike triterpenoids from *Ganoderma lucidum*, investigations of the lipid

components from *Ganoderma lucidum* are far less. It has been reported that ergosterol and its ester were found in *Ganoderma lucidum* with concentrations ranged from 0.8 to 1.6 mg/g and ergosteryl esters accounted for 3.6% to 41.9% of the total ergosterol with respect to different parts of *Ganoderma lucidum* [60]. In addition to the sterol component, approximately 20 kinds of fatty acids have been identified in *Ganoderma lucidum* lipid extracts so far and the major components include linoleic acid, oleic acid, palmitic acid and stearic acid. Contents of these acids varied from 4.97% to 76.74% depending on extraction methods and cultivation conditions [61;62]. More recently, two 19-carbon fatty acids which are nonadecanoic acid and cis-9-nonadecenoic acid has been isolated and identified in a *Ganoderma lucidum* lipid extract [8]. However, many of the lipid components remain unknown, which may need further isolation and identification.

GC-MS is a well accepted approach for identification of lipids and has been used exclusively in identifying fatty acids in *Ganoderma lucidum* [61-63]. However, the inability of GC-MS to retrieve tested samples also limits its application when subsequent bioactivities investigations on the single component of the mixture are needed. Development of LC-MS methods which can subsequently retrieve and separate a mixture of lipid compounds is therefore necessary to aid in the lipids-associated bioactivity investigations.

2.2.2 Bioactivities of *Ganoderma lucidum* Lipids in Cell Culture

Despite the limited studies on the lipid composition in *Ganoderma lucidum*, significant bioactivity of *Ganoderma lucidum* lipids has been reported in cultured human carcinoma cells. A lipids extract from *Ganoderma lucidum* has been reported to be a potential immune reaction modulator by regulating hepatic lipid metabolism as the extract specifically transactivate the PPARα in HepG2 cells [21]. *Ganoderma lucidum* lipids were also found to induce apoptosis in THP-1human leukemia cells with activation of

caspase through JNK1/2 activation and ERK1/2 and Akt inhibition [9]. Another mixture containing C-19 fatty acids was reported to inhibit cell proliferation and inducing apoptosis in HL-60 cells [8]. However, the exact lipid compositions in most of the reported extracts were not stated. The literature is also sparse on effects of *Ganoderma lucidum* lipids on other carcinoma cell.

CHAPTER 3 GANODERMA LUCIDUM TRITERPENOID EXTRACT INDUCES APOPTOSIS IN HUMAN COLON CARCINOMA CELLS (CACO-2)

Preface

Selected portion of Chapter 3 have been published in the following publication

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3.1 Introduction

As previously mentioned in Chapter 2, triterpenoids extracted or isolated from *Ganoderma lucidum* have been reported to be responsible for many of the pharmaceutical activities of *Ganoderma lucidum* [7]. So far, hundreds of triterpenoids have been found in *Ganoderma lucidum* and many more continued to be discovered [22;64;65]. Two major types of triterpenoids in *Ganoderma lucidum* are ganoderic acids (C30) and lucidenic acids (C27) and the total triterpenoid content in *Ganoderma lucidum* varies from 0.6 to 11 mg/g dry powder [66;67]. These triterpenoids were reported to influence metabolic states including exhibiting anti-diabetic properties and regulating inflammatory pathways in cell culture [2;37;68]. Triterpenoids from *Ganoderma lucidum* also possess significant chemo-preventative potential. Ganoderiol F (30 μM) was found to induce senescence in Hep-G2 cells [52]. Ganoderic acid Mf and S (0-97.7 μM) were reported to suppress cell growth of HeLa cells through apoptotic cell death [50]. Ganoderic acid T (24.20 μM) was shown to possess anti-metastasis effect on 95-D cells [54]. Multiple regulating mechanisms mediated by triterpenoid from *Ganoderma lucidum* on different colon carcinoma cells were also reported. Ganoderic acid Me was shown to induce apoptosis in HCT-116 cells with a LC50 of 36.9 μM [49]. While ganodermanontriol (0-80 μM) inhibited cell growth in HT-29 cells through down regulation of β-catenin signaling

which binds to T-cell Factor / Lymphocyte Enhancer binding Factor (Tcf/Lef) and increases transcriptional activation of cyclin D1, c-myc and proliferator-activated reporter δ [69]. Triterpenoid extracts of *Ganoderma lucidum* also exhibited cytotoxic effects on colon carcinoma cells by distinct molecular mechanisms. A *Ganoderma lucidum* extract containing a mixture of lanostanoid triterpenes (0.25 mg/mL) was found to induce autophagic cell death in HT-29 cells, while another triterpenoid enriched extract induced apoptosis in SW620 cells at a concentration of 50 μM [48;70]. However, content and composition of triterpenoids in these extracts were not reported. The objective herein is to use a bioassay-guided approach to identify *Ganoderma lucidum* fractions that are cytotoxic and induce apoptosis in Caco-2 cells.

3.2 Materials and Methods

3.2.1 Preparation of *Ganoderma lucidum* Fractions

Ganoderma lucidum was purchased locally (slice of *Ganoderma lucidum*, *chizhi*, origin from China), ground and refluxed with 500 mL 95% ethanol (1:20, w/v) for 3.5 h and repeated three times. The extraction was modified based on a refererence extraction method [71]. The crude extract was filtered, evaporated and lyophilized for further fractionation. A flash column chromatography system (combiflash companion, Teledyne Isco, Inc., Lincoln, NE, USA) coupled with a reversed phase C18 column (43 g, 40- 63 μm particle size) (RediSep Rf, Teledyne Isco, Inc., Lincoln, NE, USA) was employed for isolating triterpenoid enriched extracts. Gradient elution with a flow rate of 5 mL/min was applied to obtain four fractions. Fraction 1 was eluted with 35 % - 61 % ethanol, fraction 2 with 61 % - 75 % ethanol, fraction 3 with 75 % - 100 % ethanol and fraction 4 with 100 % ethanol. Each fraction was concentrated under vacuum and lyophilized. The triterpenoid enriched fractions (fraction 2 and 3) described below were dissolved in

culture media and passed through a 0.2 μm filter (Millex GP, Millipore, Billerica, MA, USA) for bioactive testing.

3.2.2 HPLC-ESI-MS Analysis

A Dionex Ultimate 3000 RSLC system (Dionex Corporation, Sunnyvale, CA, USA) coupled with Bruker amaZonX IonTrap Mass Spectrometer (Bruker Daltonics Inc., Billerica, MA, USA) was used for LC-MS analysis. For separation and identification, a Phenomenex Kinetex reversed phase C-18 column (3.0 mm × 100 mm, 2.6 μm particle size) (Phenomenex Inc., Torrance, CA, USA) was used. The flow rate was 0.4 mL/min, sample injection volume was 5 μL and detection wavelength was set at 254 nm. The mobile phase consisted of 1% acetic acid (A) and acetonitrile (B) and gradient conditions were set as followed: for fraction 2, 0 - 35 min, 25% B - 35% B; 35 - 45 min, 35% B; for fraction 3, 0 - 35 min, 35% B - 95% B; 35 - 40 min, 95% B - 100% B; 40 - 45 min, 100% B. For ESI-MS detection, the capillary voltage was set at -4500 V. Flow rate of dry gas was 8.0 L/min and dry temperature was set at 250 ºC. Both positive and negative ionization were used and scanning mass spectra focused on m/z range of 70 -1500 m/z. For tandem MS, a scheduled precursor list was used to fragment the most abundant precursor at specific time points.

3.2.3 Cell Culture

Caco-2 cells were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Caisson Laboratories, Inc., North Logan, UT, USA) and supplemented with 10% fetal bovine serum (Hyclone UK Ltd., Cambridge, UK), 100 units/mL of penicillin /streptomycin (Gibco, Invitrogen, Burlington, Canada) in a humidified atmosphere of 5% CO_2 at 37 ºC. Cell were maintained at a concentration between 2×10^5 and 1×10^6

cells/mL, cells were subcultured every 3 - 4 days by total replacement of media using 0.25% (w/v) trypsin - 0.53 mM ethylenediaminetetraacetic acid solution (Gibco, Invitrogen). Viable cells were assessed by the Guava Flow cytometry using 380 μL of ViaCount reagent (Guava Technologies, Inc., Hayward, CA, USA) for every sample (20 μL) for 5 min and data acquired using CytoSoft software equipped with the ViaCount module.

3.2.4 Cell Viability Assay

Caco-2 cells were seeded at a concentration of 2×10^4 cells/mL in 96-well plates. After 24 h, cells were treated with 100 μL/well either fraction 2 or 3 at concentrations between 0.1 and 1 mg/mL for 72 h, which were dissolved with 0.4% dimethyl sulfoxide (DMSO) in DMEM. Untreated cells acted as controls. After treatment, the media was removed and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, St Louis MO, USA) dissolved in DMEM was added at a concentration of 0.5 mg/mL. Cells were incubated in the dark for 4 h to allow formation of formazan crystals. To solubilize the crystals, 100 μL/well of SDS (10%) in HCl (0.01 N) was added and incubated overnight as previous described [72]. The optical density was measured at absorbance of 570 nm (sample absorbance) and 650 nm (reference absorbance) by a microplate reader (Multiskan Spectrum, Thermo Electron Corporation, Waltham, MA, USA). Cell viability was calculated as [(mean sample absorbance – mean reference absorbance) / (mean control absorbance – mean reference absorbance)] × 100%.

3.2.5 Cell Cycle Analysis

Fraction 2 and 3 were added to Caco-2 cells (2×10^4 cells/mL) in 6-well plates at their respective LC50 concentrations obtained from the MTT assay. Cells were incubated at 37 ºC in a 5% CO_2 humidified incubator for 24, 48 and 72 h. Untreated cells acted as

controls at each respective time points. After treatment, non-adherent cells in culture media were collected by centrifugation at 2500 g for 5 min. The adherent cells were trypsinized for 10 min, mixed with 2 mL media and centrifuged at 500 g for 7 min. Cell pellets were further washed with phosphate buffered saline (PBS) twice followed by centrifugation to remove PBS. Cell pellets of both non-adherent and adherent cells were combined and fixed with 70% ice-cold ethanol overnight at -15 ºC. Fixed cells were centrifuged at 500 g for 7 min to remove ethanol and washed with PBS twice. After removing PBS by centrifugation, 1 mL of PBS containing propidium iodide (PI, 50 µg/mL, Sigma) and RNase A (100 units /mL, Applichem Inc., St. Louis, MO, USA) were added and incubated in the dark for 30 min at room temperature. Samples were analyzed by Guava PCA flow cytometry with CytoSoft software (Guava Technologies Inc.) as previous described [73].

3.2.6 Caspase Apoptotic Assay

Cells were seeded in 6-well plates at a cell concentration of 2×10^4 cells/mL. After 24 h, cells were treated with fraction 3 at the LC50 concentration for 48 h. Untreated cells acted as controls. After treatment, non-adherent cells in culture media were collected by centrifugation at 2500 g for 5 min. The adherent cells were detached by trypsinization and centrifuged at 300 g for 7 min. The cell pellets were combined and washed with PBS twice. Cells were suspended in PBS and adjusted to a concentration of 5×10^5 cells/mL by adding 1× apoptotic wash buffer (Guava Technologies Inc.). Five µL of 20 × sulforhodamine - valyl - alanyl - fluoromethyl - ketone (SR-VAD-FMK) reagent was added to each sample and incubated for 1 h in the dark at 37 ºC in a 5% CO_2 incubator. The cells were washed with 1× apoptotic wash buffer twice, centrifuged and suspended in 100 µL 1× apoptotic wash buffer. After the first staining, a second staining with 5 µL of caspase 7-amino-actinomycin D (7-AAD) (Guava Technologies Inc.) was employed to

each sample for 10 min at room temperature. The sample was finalized to a 200 µL sample volume with 1× apoptotic wash buffer and analyzed on Guava PCA flow cytometry.

3.2.7 Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

To allow induction of late apoptotic cells, fraction 3 was added at the LC50 concentration for 72 h in 6-well plates at a seeding concentration of 1×10^4 cells/mL. Untreated cells acted as controls. After treatment, non-adherent cells were collected by centrifugation at 2500 g for 5 min and adherent cells were harvested by trypsinization and centrifuged at 300 g for 7 min. Cell pellets were combined and washed with PBS. After removing PBS by centrifugation, cells were fixed with 4% paraformaldehyde at 4 ºC for 45 min followed by 70% ice-cold ethanol overnight at -15 ºC. Fixed cells were centrifuged to remove ethanol and washed with 1 mL of the wash buffer (Guava Technologies Inc.) three times. The process of cell staining was conducted according to the manufacturer's instructions. Briefly, cells were treated with 25 µL of the DNA Labeling Mix Reagent (Guava Technologies Inc.) for 90 min at 37 ºC followed by washing with rinsing buffer and centrifugation. The cells were suspended in 50 µL of the Anti-BrdU Staining Mix Reagent (Guava Technologies Inc.) and incubated for 30 min at room temperature in the dark. At the end of the process, each sample was diluted with 150 µL rinsing buffer and acquired by a Guava PCA system flow cytometry.

3.2.8 Statistical Analysis

A one-way analysis of variance (ANOVA) with Duncan post hoc comparison of means and an independent-samples T test were used. Significance was determined at $p < 0.05$ using the SPSS statistical software (v12.0, Chicago, IL, USA). HPLC-ESI-MS

analysis was conducted with three replicate in two separate experiments. The MTT assay, cell cycle analysis, caspase apoptotic assay and TUNEL assay were determined in three separate experiments with three replicate.

3.3 Results

3.3.1 Triterpenoid Profiles by HPLC-ESI-MS

Four fractions separated from flash chromatography had recoveries of 6.32%, 13.24%, 4.35% and 1.24% for fraction 1, 2, 3 and 4 repectively calculated based on their weight of powder after lyophilizaiton. Only fraction 1, 2 and 3 had UV absorption in the HPLC analysis while fraction 1 cannot be identified as any known components based on HPLC-ESI-MS analysis. Representative HPLC chromatographs of fraction 2 and 3 are shown in **Figure 2** panels (a) and (b) respectively. Fraction 2 contained 12 major triterpenoids, including ganoderic acid I (peak 1), ganoderic acid C2 (peak 2), ganoderic acid C6 (peak 3), ganoderic acid G (peak 4), ganoderic acid ζ (peak 5), ganoderic acid B (peak 6), ganoderenic acid K (peak 7), ganoderic acid K (peak 8), ganoderic acid A (peak 9), ganoderic acid H (peak 10), ganoderenic acid D (peak 11) and ganoderic acid D (peak 12). Fraction 3 consisted of three major compounds and two were identified as ganoderic acid DM (peak 19) and ganoderic aldehyde TR (peak 21). Additionally, two other minor components were identified as lucidenic acid D1 (peak 15) and ganoderic acid Z (peak 17). The maximum absorbent wavelengths of tested compounds determined by photodiode array detector and fragmentation patterns generated from tandem mass spectrometry (MS) in both positive mode and negative mode were utilized for identification by comparing fragmentation patterns, retention times and molecular weights to published references [24;32;74-79]. Representative MS fragments of compounds in fraction 2 and 3 extracts are listed in **Table 2**. Since the fragmentation patterns of compounds corresponding to peak 13, 14, 16, 18 and 20 did not match any

published references, these compounds were considered as unknown compounds. They were subsequently isolated into individual compounds and identified by NMR analysis as ganolucidic acid E (peak 13), lucidumol A (peak 14), ganodermanontriol (peak 16), 7-oxo-ganoderic acid S (peak 18) and 15-hydroxy-ganoderic acid Z (peak 20) in charpter 6. Chemical structures of the components identified in fraction 2 and 3 in this charptor are illustrated in **Figure 3**.

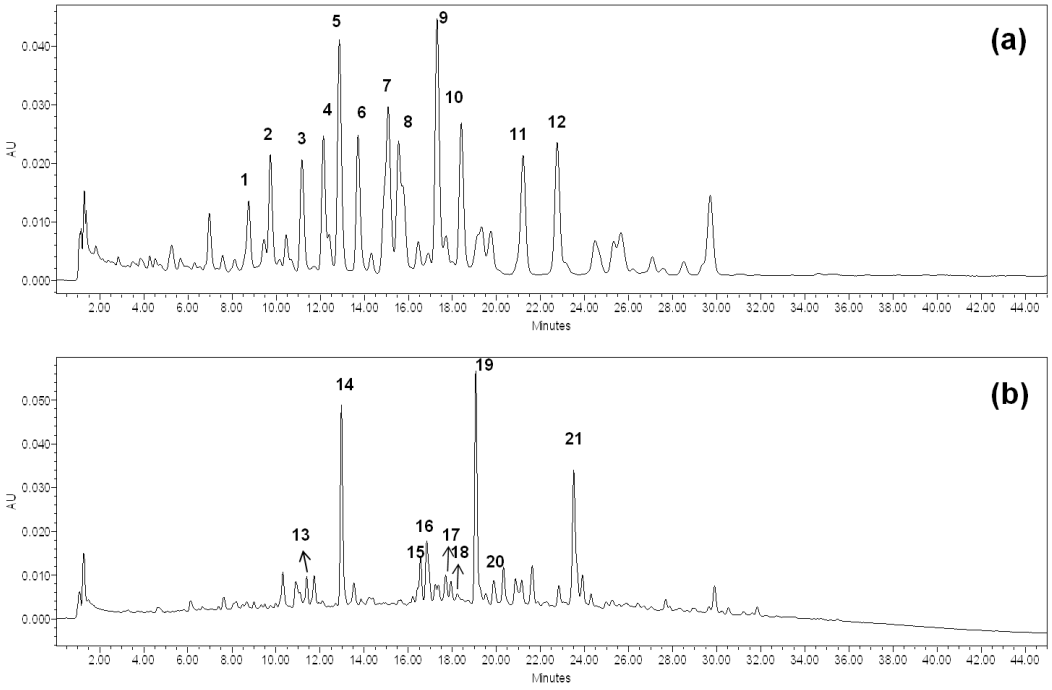


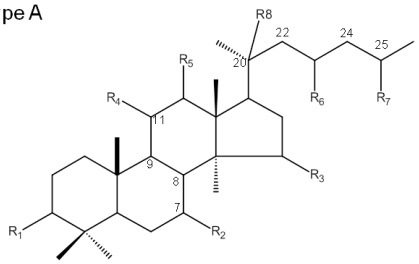
Figure 2. Representative HPLC chromatographs of fraction 2 (panel a) and fraction 3 (panel b) from *Ganoderma lucidum*. 1, ganoderic acid I; 2, ganoderic acid C2; 3, ganoderic acid C6; 4, ganoderic acid G; 5, ganoderic acid ζ; 6, ganoderic acid B; 7, ganoderenic acid K; 8, ganoderic acid K; 9, ganoderic acid A; 10, ganoderic acid H; 11, ganoderenic acid D; 12, ganoderic acid D; 13*, ganolucidic acid E; 14*, lucidumol A; 15, lucidenic acid D1; 16*, ganodermanonotriol; 17, ganoderic acid Z; 18*, 7-oxo-ganoderic acid S; 19, ganoderic acid DM; 20*, 15-hydroxy-ganoderic acid Z; 21, ganoderic aldehyde TR.*, compounds were identified in chapter 6.

Table 2. Representative MS fragments of major compounds in *Ganoderma lucidum* fraction 2 and 3 extracts.

Peak No.	Compound	Representative MS fragments
1	ganoderic acid I	531.0$[M-H]^-$, 513.3$[M-H-H_2O]^-$, 1063.7$[2M-H]^-$
2	ganoderic acid C2	517.3$[M-H]^-$, 499.3$[M-H-H_2O]^-$, 437.4$[M-H-H_2O-CO_2]^-$, 1035.8$[2M-H]^-$
3	ganoderic acid C6	529.2$[M-H]^-$, 511.3$[M-H-H_2O]^-$, 467.4$[M-H-H_2O-CO_2]^-$, 437.4$[M-H-H_2O-CO_2-CH_2O]^-$,

		1059.6[2M-H]$^-$
4	ganoderic acid G	531.3[M-H]$^-$, 513.3[M-H-H_2O]$^-$, 469.3[M-H-H_2O-CO_2]$^-$, 1063.8[2M-H]$^-$
5	ganoderic acid ζ	513.3[M-H]$^-$, 495.3[M-H-H_2O]$^-$, 399.2[M-H-$C_5H_6O_3$]$^-$, 1027.8[2M-H]$^-$
6	ganoderic acid B	515.3[M-H]$^-$, 497.3[M-H-H_2O]$^-$, 453.3[M-H-H_2O-CO_2]$^-$, 1031.5[2M-H]$^-$
7	ganoderenic acid K	571.3[M-H]$^-$, 553.4[M-H-H_2O]$^-$, 1143.7[2M-H]$^-$
8	ganoderic acid K	573.3[M-H]$^-$, 555.3[M-H-H_2O]$^-$, 513.3[M-H-$C_2H_4O_2$]$^-$, 511.3[M-H-H_2O-CO_2]$^-$, 1147.7[2M-H]$^-$
9	ganoderic acid A	515.3[M-H]$^-$, 497.3[M-H-H_2O]$^-$, 479.3[M-H-2H_2O]$^-$, 435.4[M-H-2H_2O-CO_2]$^-$, 1031.6[2M-H]$^-$
10	ganoderic acid H	571.1[M-H]$^-$, 553.3[M-H-H_2O]$^-$, 511.3[M-H-$C_2H_4O_2$]$^-$, 1143.7[2M-H]$^-$
11	ganoderenic acid D	511.3[M-H]$^-$, 493.3[M-H-H_2O]$^-$, 1023.4[2M-H]$^-$
12	ganoderic acid D	513.3[M-H]$^-$, 495.0[M-H-H_2O]$^-$, 451.3[M-H-H_2O-CO_2]$^-$, 1027.5[2M-H]$^-$
13	unknown	485.6[M+H]$^+$, 483.3[M-H]$^-$
14	unknown	473.4[M+H]$^+$, 471.6[M-H]$^-$
15	lucidenic acid D1	471.4[M+H]$^+$, 469.5[M-H]$^-$, 939.6[2M-H]$^-$, 353.4[M-$C_6H_{12}O_2$-H]$^-$
16	unknown	531.8[M-H]$^-$
17	ganoderic acid Z	457.4[M+H]$^+$, 439.3[M+H-H_2O]$^+$, 383.5[M+H-H_2O-C_3H_4O]$^+$
18	unknown	471.3[M+H]$^+$
19	ganoderic acid DM	469.4[M+H]$^+$, 937.3[2M+H]$^+$, 466.9[M-H]$^-$, 935.6[2M-H]$^-$, 326.3[M- $C_8H_{13}O_2$-H]$^-$
20	unknown	469.3[M+H]$^+$
21	ganoderic aldehyde TR	453.3[M+H]$^+$, 435.3[M+H-H_2O]$^+$, 395.3[M+H-H_2O-C_3H_6O]$^+$, 327.23[M+H –$C_8H_{14}O$]$^+$

Type A



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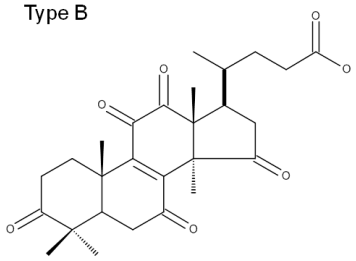


compound	fraction	type	R1	R2	R3	R4	R5	R6	R7	R8	double bond
1	2	A	βOH	βOH	O	O	H	O	COOH	αOH	8,9;
2	2	A	βOH	βOH	αOH	O	H	O	COOH	H	8,9;
3	2	A	βOH	O	O	O	βOH	O	COOH	H	8,9;
4	2	A	βOH	βOH	O	O	βOH	O	COOH	H	8,9;
5	2	A	βOH	O	O	O	H	αOH	COOH	H	8,9; 24,25;
6	2	A	βOH	βOH	O	O	H	O	COOH	H	8,9;
7	2	A	βOH	βOH	O	O	βOAc	O	COOH	-	8,9; 20,22;
8	2	A	βOH	βOH	O	O	βOAc	O	COOH	H	8,9;
9	2	A	O	βOH	αOH	O	H	O	COOH	H	8.9;
10	2	A	βOH	O	O	O	βOAc	O	COOH	H	8,9;
11	2	A	O	βOH	O	O	H	O	COOH	-	8,9; 20,22;
12	2	A	O	βOH	O	O	H	O	COOH	H	8,9;
15	3	B	-	-	-	-	-	-	-	-	-
17	3	A	βOH	H	H	H	H	H	COOH	H	8,9; 24,25;
19	3	A	O	O	H	H	H	H	COOH	H	8,9; 24,25;
21	3	A	O	H	αOH	H	H	H	CHO	H	7,8; 9,11;

Figure 3. Chemical structures of representative compounds in *Ganoderma lucidum* fraction 2 and 3 extracts, numbers under compound refers to the Table 2 column 1.

3.3.2 MTT Cytotoxicity Assay and Cell Cycle Distribution

Representative dose-response relationships of fraction 2 and 3 extracts on Caco-2 cell viability are shown in **Figure 4.** The LC50 values were determined by plotting cell viabilities against log concentrations (from 0.1 to 1mg/mL, graph not shown) and linear equations of $y = -60.236\ x + 33.076$ ($R^2 = 0.9515$) (fraction 2) and $y = -97.085\ x + 5.655$ ($R^2 = 0.9238$) (fraction 3) were determined. The LC50 value of fraction 2 and 3 extracts on Caco-2 cells was calculated to be 0.528 ± 0.078 and 0.348 ± 0.032 mg/mL respectively. The representative dose-response relationship of fraction 1 extract on

Caco-2 cell viability is shown in Apendix 2 and the ones of crude extract and fraction 4 are discussed in chapter 4.

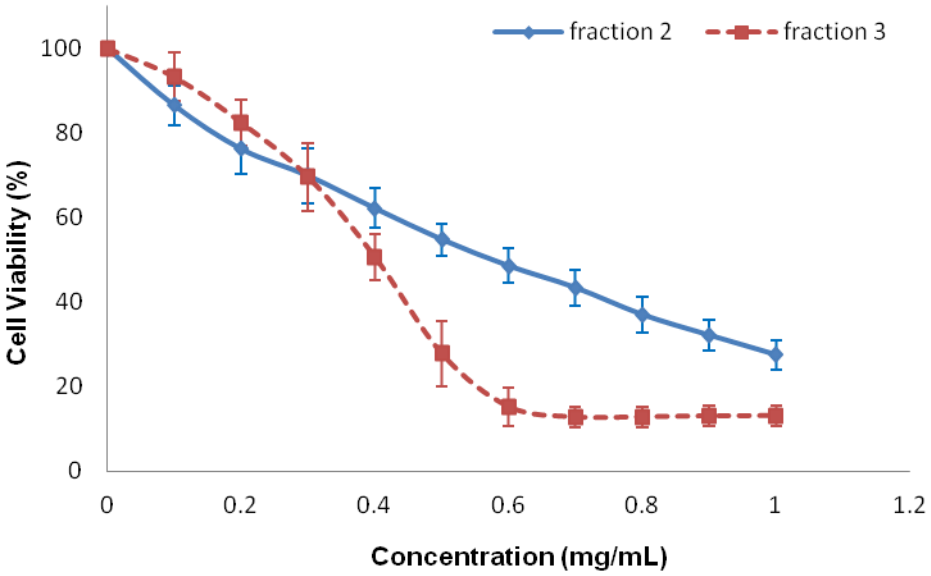


Figure 4. Cell viability of fraction 2 and 3 treated Caco-2 cells for 72h and assessed using the MTT assay. Values are expressed as mean ± SD (percentage of untreated control cells) of three separate experiments with three replicate.

The corresponding cell cycle distributions of fraction 2 and 3 treatment at their respective LC50 concentration for 24, 48 and 72 h are shown in **Table 3**. Treatment of fraction 2 and 3 both significantly ($p < 0.05$) induced cell cycle arrest at G2/M phase for all the time periods and maximum accumulation (6.43 % for fraction 2 and 9.76 % for fraction 3) for 48 h treatment. The results also indicated a significant ($p < 0.05$) reduction of cell percentage at G0/G1 phase for all the time periods on Caco-2 cells treated by both fractions and maximum reduction of 10.94% for fraction 2 and 21.34% for fraction 3 were observed after 72 h. Conversely, cells treated with fraction 3 were found to be significantly ($p < 0.05$) increased (6.46% and 15.95%) at the sub-G1 phase for 48 and 72 h, indicating DNA fragmentation, while fraction 2 did not show differences in this phase compared to the control cells.

Table 3. Cell cycle analysis of Caco-2 cells with treatment of fraction 2 and 3 at their respective LC50 concentration obtained by MTT assay. Values are expressed as mean ± SD. An asterisk represents significant differences ($p < 0.05$) in the same phases within the same period compared to control values.

			Sub-G1	G0/G1	S	G2/M
Fraction 2	24 hr	control	5.50±0.74	36.70±1.41	25.23±3.86	37.28±3.33
		sample	4.13±0.17*	31.64±2.32*	20.50±2.12*	42.20±2.38*
	48 hr	control	3.23±0.67	45.27±0.70	16.27±3.70	34.87±2.38
		sample	3.17±1.11	32.93±4.19*	22.53±3.63*	41.30±2.57*
	72 hr	control	2.65±0.44	49.30±1.78	15.88±1.32	32.88±1.67
		sample	3.53±0.59*	38.36±0.86*	20.39±4.17*	36.58±0.96*
Fraction 3	24 hr	control	2.30±0.36	38.40±3.14	20.37±1.36	38.53±1.94
		sample	3.27±1.80	33.23±4.91*	17.7±3.30*	41.07±2.33*
	48 hr	control	3.27±0.31	44.80±6.61	22.50±2.48	32.97±0.50
		sample	9.73±2.02*	29.40±0.98*	18.53±1.18*	42.73±3.01*
	72 hr	control	2.83±0.60	46.47±1.50	18.97±0.32	31.50±2.41
		sample	18.77±4.37*	25.13±3.15*	20.17±2.67*	37.82±5.09*

3.3.3 Detection of Apoptosis by Caspase Apoptotic and TUNEL Assays

To confirm the induction of apoptosis by fraction 3, caspase apoptotic assay was conducted. The different stages of apoptosis induced by treatment of fraction 3 extract on Caco-2 cells are shown in **Figure 5**. As described previously [80], mid-apoptotic cells are stained positively with SR-VAD-FMK but not with the membrane integrity indicator 7-AAD. The late apoptotic cells are stained positively by both SR-VAD-FMK and 7-AAD while viable cells are not stained with neither. Dead cells would not be stained by SR-VAD-FMK but would be positively stained with 7-AAD. Compared to the control, treatment of fraction 3 for 48 h significantly ($p < 0.05$) increased mid-apoptotic cells (6.27%) and late-apoptotic cells (10.59%). Correspondingly, viable cells from fraction 3

treatment significantly ($p < 0.05$) decreased 15.67% compared to the control value. The percentage of dead cells did not show significant differences between treatment and control.

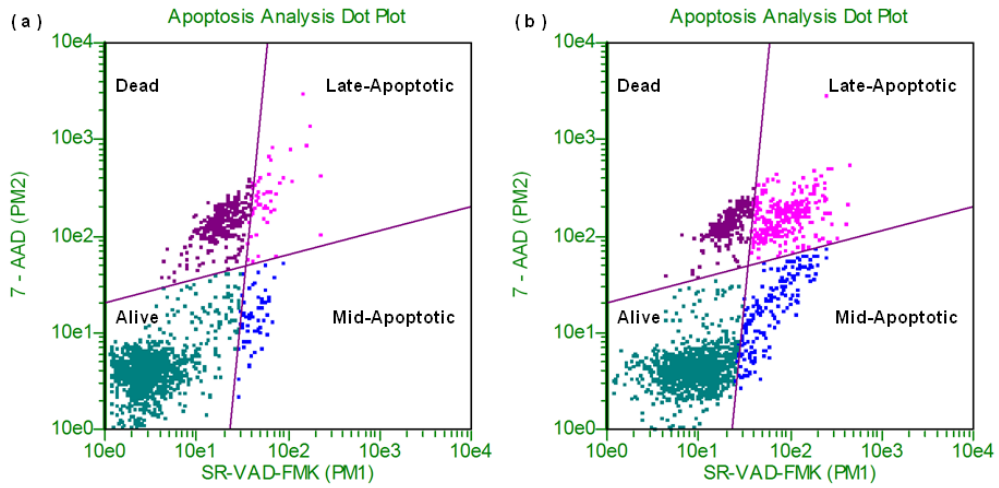


Figure 5. Representative flow cytometry analysis of caspase activity of fraction 3 treated Caco-2 cells. Caco-2 cells were treated by fraction 3 extract at the LC50 concentration determined by the MTT analysis for 48 h (panel b). Untreated cells acted as controls (panel a). Quadrant analysis of control cells indicated 74.84 ± 2.94% viable cells compared to 59.17 ± 5.45%* with fraction 3 treatment. Dead cells were 14.37 ± 3.72% of control and 13.11 ± 0.58% of fraction 3 treatment. Mid-apoptotic cells were 5.46 ± 1.15% of control and 11.73 ± 2.05%* of fraction 3. Late apoptosis cells were 5.44 ± 1.38% of control and 16.03 ± 2.45%* of fraction 3. SR-VAD-FMK is labeled on the x-axis, and 7-AAD is labeled on the y-axis. An asterisk represents a significant difference ($p < 0.05$) compared to the corresponding control values.

To quantify the apoptotic fragment induced by treatment of fraction 3, TUNEL assay was used and analyzed by flow cytometry. The DNA fragments generated by the induction of apoptosis were immuno-stained positively by a fluorescence dye FITC. Results of this assay are shown in **Figure 6**. Fraction 3 treatment for 72 h resulted in a significant ($p < 0.05$) increase in apoptotic cells (27.94%) and a corresponding decrease in non-apoptotic cells compared to the untreated control cells.

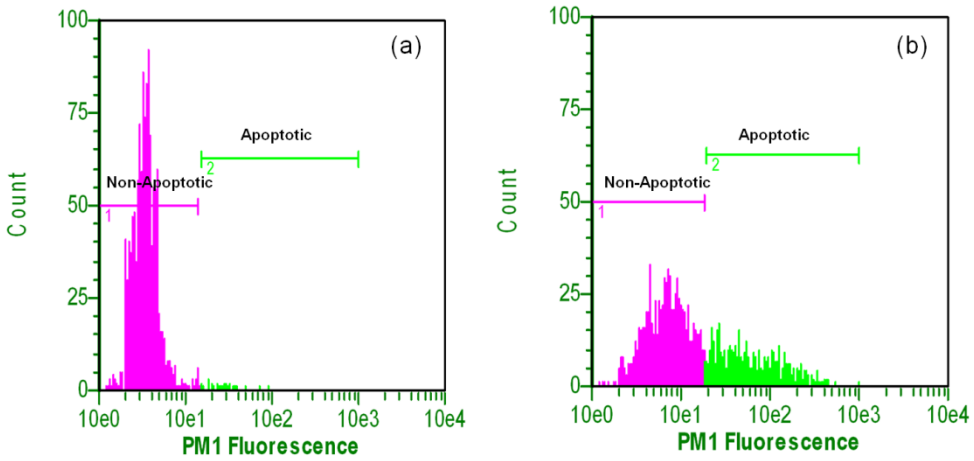


Figure 6. TUNEL apoptotic analysis of fraction 3 extract-treated Caco-2 cells for 72 h at the LC50 concentration. Data are expressed as mean ± SD of three replicate with three separate experiments. Analysis of control cells **(a)** resulted in 97.97 ± 0.68% non-apoptotic, and 2.03 ± 0.68% were apoptotic. Fraction 3 treated cells **(b)** were found to have 70.03 ± 3.03%* non-apoptotic, and 29.97 ± 3.03%* apoptotic. An asterisk represents a significant difference ($p < 0.05$) compared to the corresponding control values.

3.4 Discussions

Two distinct triterpenoid containing fractions were prepared from *Ganoderma lucidum* based on polarity. Both extracts were found to be cytotoxic and were dependent on the concentration of the extracts. LC50 of fraction 2 was 0.528 ± 0.078 mg/mL and fraction 3 was 0.348 ± 0.032 mg/mL. Fraction 2 contained ganoderic acids ζ, A, B, C2, C6, D, G, H, I, K and ganoderenic acids D and K and were more polar compared to fraction 3 which contained lucidenic acid D1, ganoderic acid Z, ganoderic acid DM and ganoderic aldehyde TR. The cytotoxic properties reported here are consistent with results obtained in other cell lines such as human hepatoma Huh-7 cells and MCF-7 cells that have reported LC50 values of 0.45 and 0.22 mg/mL respectively [45;81]. Similar compounds found in ginseng have been reported to possess a structure function effect in reducing cell viability in cultured cancer cells and cytotoxicity generally increases as the polarity decreases [82].

Both fractions caused a cell cycle arrest at G2/M phase with maximum increased cell percentages of 6.43 % and 9.76 % for fraction 2 and 3 treatment respectively. The G2/M arrest is consistent with observations in other cell lines including Huh-7 cells, HL-60 cells and RPMI8226 cells [45;83]. Further investigation of specific cyclins and cyclin-dependent kinases may help in understanding the mediation of this cell cycle arrest. Additionally, fraction 3 but not fraction 2 treatment suggested a significant accumulation of cells in the sub-G1 phase of the cell cycle, which indicated DNA fragmentation and apoptosis. The induction of apoptosis by fraction 3 treatment was further confirmed and quantified by caspase apoptotic and TUNEL assays. The positive results shown in SR-VAD-FMK staining in the caspase apoptotic assay also suggested that activation of the caspase cascade was involved in the induction of apoptosis in Caco-2 cells treated with fraction 3. Further studies are required to determine the specific caspase involved. Similar cell death mechanism was reported in another colon carcinoma cell line with triterpenoid extract treatment [48]. However, induction of apoptosis was not the only reported cell death mechanism by triterpenoids from *Ganoderma lucidum* and multiple cell death mechanisms were proposed. A triterpenoid extract (0.25 mg/mL) was reported to induce autophagic cell death in HT-29 cells with increasing levels of autophagic markers Beclin-1 and microtubule-associated protein 1 light chain 3, by the inhibition of p38 mitogen-activated protein kinase [84]. Since the compositions and content of triterpenoids in the reported extracts were not specified, the molecular mechanism responsible for the cytotoxic effects of triterpenoids from *Ganoderma lucidum* is likely cell specific and dependent on chemical structures. This is evident by the different bioactive response obtained from fraction 2, which contained triterpenoids that all have an oxyl or hydroxyl group at position C-23, and fraction 3, which contained identified triterpenoids that did not have any functional groups at C-23. Both extracts exhibited cytotoxicity but varied in apoptotic response. The side chain variability of triterpenoids is

likely to be responsible for these distinct cell cyto-activities. A structure-activity relationship study in multiple cell lines will add to the understanding of behavior of triterpenoids from *Ganoderma lucidum* in cancer cell models. Interestingly, an unknown compound possibly a triterpenoid was found to be one of the major components in fraction 3. Further isolation and purification are underway to identify the exact chemical structure of this unknown compound. The quantification of triterpenoids in the extract is challenging due to the unavailability of commercial standards and variability in maximum absorbance wavelengths of triterpenoids. Nevertheless, this chapter shows the induction of apoptosis from specific set of triterpenoids in cultured human colon carcinoma Caco-2 cells. The findings may contribute to the understanding of the diverse pharmaceutical properties of *Ganoderma lucidum* triterpenoids as chemo-preventative agents that is dependent on the chemical structure.

3.5 Conclusions

An ethanolic extraction of *Ganoderma lucidum,* a medicinal mushroom, was fractioned into four extracts by flash chromatography. Only fraction 2 and 3 contained triterpenoids. Fraction 2 was less polar and contained ganoderic acids ζ, A, B, C2, C6, D, G, H, I, K and ganoderenic acids D and K, all with presence of oxyl or hydroxyl groups at position C23. Fraction 3 contained ganoderic acid DM, ganoderic aldehyde TR and an unknown compound. None of identified compounds has functional groups at C23. Both fraction 2 and 3 inhibited Caco-2 cells in a dose dependent manner. The LC50s of fraction 2 and 3 were 0.528 ± 0.078 and 0.348 ± 0.032 mg/mL. A significant accumulation of sub - G1 cells (18.77 ± 4.37 %) was found with fraction 3 but not with fraction 2. TUNEL assay resulted in 29.97 ± 3.03 % apoptotic cells and caspase apoptotic assay showed 11.73 ± 2.05 % mid-apoptotic cells and 16.03 ± 2.45 % late apoptotic cells. The caspase apoptotic assay indicated an involvement of caspase enzyme family

mediation. Two fractions were cytotoxic. Fraction 2 caused a G2/M arrest while fraction 3 induced apoptosis. These differences are likely a result of the polarity of the triterpenoids.

CHAPTER 4 GANODERMA LUCIDUM EXTRACT AND ITS LIPID ENRICHED FRACTION INDUCE DIFFERENTIATION IN CACO-2 HUMAN COLON CARCINOMA CELLS

Preface

Selected portion of Chapter 4 are ready to be submitted as:

Ruan, W.; Popovich, D. G. *Ganoderma lucidum* extract and its lipid enriched fraction induce differentiation in Caco-2 human colon carcinoma cells. *xxx*. **2013**.

4.1 Introduction

Colorectal cancer is the third most common cancer with approximately 1.2 million new cases diagnosed each year and the fourth leading cause of cancer deaths with more than 600 000 deaths each year worldwide [85]. Studies on colorectal cancer and its prevention and treatment by traditional medicine have garnered increasing interests nowadays. *Ganoderma lucidum*, as one of the most extensively used and pharmaceutical effective medicinal herbs, has been reported to inhibit cell growth in colon carcinoma cells through inducing apoptotic and autophagic cell deaths, most of which is due to its triterpenoid contents [86;87]. However, effects of *Ganoderma lucidum* lipids on colon carcinoma cells remains unclear, though a lipid extract of *Ganoderma lucidum* has been reported to regulate hepatic lipid metabolism in human carcinoma hepatic cells (HepG2) and its C-19 fatty acids to induce apoptosis in human leukemia cells (HL-60) [8;21].

Lipids such as fatty acids have been strongly suggested as adjunctive chemotherapeutic agents for colon cancer due to their diverse anti-neoplastic properties to promote apoptosis [88]. For instant, Docosahexaenoic acid (DHA) has been reported to reduce cell viability and promote apoptosis possibly through altering gene expression and activating caspase and poly ADP-ribose polymerase (PARP) in colon carcinoma cells

[89;90]. These bioactivities in colon carcinoma cells vary with respect to a different lipid composition. Unlike DHA, short chain fatty acids such as butyrate, mostly reduced the cell viability by promoting colorectal cell differentiation. Though the exact mechanisms remain to be elucidated, it is believed that this action required activation of JNK and PKC pathways and was probably through inhibiting histone deacetylase which subsequently allowed transcription factors to activate differentiation-related genes [91-93]. Induction of differentiation could be of clinical significance to regain normal cell control from malignant phenotype as differentiated cells may serve as transition stage from proliferation to cell death with limited proliferative capacity and life span [94;95]. Therefore, based on the above understanding, we believed that *Ganoderma lucidum* lipids might possess a similar adjunctive colorectal chemotherapeutic potential by influencing the cell differentiation and apoptosis. Hence, this chaptor was to investigate the effects of a lipid-enriched fraction of *Ganoderma lucidum* extract on viability and differentiation on human colon carcinoma Caco-2 cells and possible signaling involvement.

4.2 Materials and Methods

4.2.1 Extracts Preparation

Dried *Ganoderma lucidum* was purchased locally (slice of *Ganoderma lucidum*, *chizhi*, origin from China), grounded and extracted with 95% ethanol thrice. The alcohol was evaporated under vacuum and the extract was lyophilized. The extract was then fractioned by a flash column chromatography system (Combiflash companion) with a reversed phase C18 column (43 g, 40- 63 μm particle size) (RediSep Rf, Teledyne Isco, Inc.) to give four fractions as described in Chapter 3 [96]. Fraction 1 was eluted with 35 % - 61 % ethanol, fraction 2 with 61 % - 75 % ethanol, fraction 3 with 75 % - 100 % ethanol and fraction 4 with 100 % ethanol. Each solvent mixture was evaporated under vacuum and lyophilized.

4.2.2 Determination of Lipid Composition

The non-esterified fatty acid content was quantified by a colorimetric enzyme assay with a commercial kit (NEFA C, Wako Pure Chemical Industries, Ltd. Osaka, Japan). The crude extract and the four fractions were dispersed into deionized water by sonication to a concentration of 1 mg/mL and 50 µL was used for each sample according to the manufacture's instruction.

A Dionex Ultimate 3000 RSLC system (Dionex Corporation) coupled with Bruker amaZonX IonTrap Mass Spectrometer (Bruker Daltonics Inc.) was used to determine the specific lipid composition of fraction 4. A Phenomenex Kinetex reversed phase C-18 column (3.0 mm × 100 mm, 2.6 µm particle size) (Phenomenex Inc.) was used. The mobile phase consisted of 1% acetic acid (A) and acetonitrile (B). The flow rate was set at 0.4 mL/min and gradient conditions were set as followed: 0 - 35 min, 35% B - 95% B; 35 - 40 min, 95% B - 100% B; 40 - 55 min, 100% B. For ESI-MS/MS detection, a capillary voltage of -4500 V, a dry gas flow rate of 8.0 L/min and a dry temperature of 250 ºC were set. The scanning mass spectra range from 70 m/z to 1200 m/z was used under both positive and negative ionization. Auto MS/MS was applied so that two of the most abundant precursor ions were fragmented at each time point with a fragment amplification power of 2.5 V. The standard solution of oleic acid from the NEFA C kit was used as an external standard. Fraction 4 was dissolved in 70% acetonitrile in a concentration of 0.6 mg/mL and 3 µL was injected for analysis.

The lipid composition of fraction 4 further went through NMR analysis for structure confirmation. A Bruker AMX 500 NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) was used. Fraction 4 (5 mg) was dissolved in deuterated chloroform ($CDCl_3$) and distortionless enhancement of polarization transfer (DEPT) ^{13}C NMR analysis was applied.

4.2.3 Cell Culture

Caco-2 cells derived from American Type Culture Collection (Manassas, VA, USA). The cells were cultured as stated in chapter 3. Viable cells were counted using a hemocytometer by staining cells with 0.04% trypan blue exclusion dye (MP Biomedicals, Solon, OH, USA). All sample treatments were dissolved with 0.4% DMSO in DMEM before tests.

4.2.4 MTT Assay

Caco-2 cells were seeded in 96-well plates (2×10^4 cells/mL) for 24 h. Cells were treated with either the crude extract or fraction 4 at concentrations between 0.1 and 1 mg/mL for 72 h. A further set of cells were cultured for 4 days to begin differentiation, and then treated with the crude extract or fraction 4 at concentration between 0.1 and 1 mg/mL for 48 h. Untreated cells acted as controls. A 0.5 mg/mL MTT solution (Sigma) was added after treatment and incubated in the dark for 4 h. SDS (10%) in HCl (0.01 N) was added and incubated overnight to dissolve the crystals. The optical density was measured at absorbance of 570 nm with a reference wavelength of 650 nm by a microplate reader (Multiskan Spectrum, Thermo Electron Corporation, Waltham, MA, USA). Cell viability was calculated as [(mean sample absorbance – mean reference absorbance) / (mean control absorbance – mean reference absorbance)] × 100% [97].

4.2.5 Cell Cycle Analysis

Cells were seeded in 6-well plates at a concentration of 2×10^4 cells/mL. The crude extract at its LC50 concentration determined by MTT assay (described below) and fraction 4 at a concentration of 0.5 mg/mL were tested for 24, 48 and 72 h respectively. Untreated cells acted as controls at each time points. Cell pellets were collected, fixed and stained as previous described in chapter 3 [98].

4.2.6 Alkaline Phosphatase Activity

Caco-2 cells were seeded in 12-well plates (2×10^4 cells/mL) for 4 days before treatment to allow cells to start differentiation. Cells were then incubated with 0.5 mg/mL of either the crude extract or fraction 4 for 48 h. After treatment, the cells were homogenized with 2% triton X-100- 1×- tris - buffer solution (triton X-100, Eastman Kodak Company, Rochester, NY, USA ; 20× tis-buffer tablet, Sigma) and centrifuged at 2500 g for 10 min at 4 ºC to obtain cell lysate. 100 µL/well of 1× p-nitrophenyl phosphate solution (20 × p- nitrophenyl phosphate tablets, Sigma) was added into 100µL/well cell lysates for 30 min at room temperature in the dark to allow the development of the yellow soluble end product p-nitrophenol. The enzyme activity was measured by determining the amount of p-nitrophenol spectrophotometrically at a wavelength of 405 nm using a microplate reader (Multiskan Spectrum). One unit of enzyme activity is defined as the activity that hydrolyzes 1 µmol p- nitrophenyl phosphate per min at 37°C. The total protein content of each sample was determined by Bradford's method using a commercial kit (Bio-rad protein assay dye reagent, Bio-Rad Laboratories Inc. Hercules, CA, USA) [99].

4.2.7 Protein Kinase Inhibitors

To determine the possible involvement of signal transduction pathway during differentiation, three protein kinase inhibitors of MEK/ERK (U0126, 10 µM, Sigma), JNK (SP600125(10 µM, Sigma) and PKC (Ro-31-8220, 10 nM, Cayman chemical, Ann Arbor, MI, USA), all of which have been reported to mediate Caco-2 differentiation, were added to the media 30 min prior to the treatment of crude extract or fraction 4 (described above) [100-102].

4.2.8 Lactase Activity

Cells were seeded, cultured and treated as described above (alkaline phosphatase activity). Cells were harvested and homogenized with 2% triton X-100 solution (Eastman Kodak Company) at 4ºC and pelleted by centrifuge at 2500 g for 10 min. The enzyme activity was determined by its catalytic hydrolysis of o-nitrophenyl-β-D-galactopyranoside (ONPG) substrate to generate a yellow end product. The reaction was carried out in 10 μL 0.1% bovine serum albumin (BSA), 50 μL 10 mM ONPG - 0.1M citrate - phosphate solution (pH 4.3, Sigma), 40 μL cell lysates at 37ºC for 30 min and stopped by adding 100 μL 1 M Na_2CO_3. The absorbance of the end product was measured at 410 nm using a microplate reader (Multiskan Spectrum). One unit of enzyme activity is defined as the activity that hydrolyzes 1 μmol ONPG per min at 37°C. The total protein content of each sample was determined by Bradford's method with the commercial kit (Bio-rad protein assay dye reagent) [99].

4.2.9 Statistical Analysis

The data were analyzed using the SPSS statistical software (v12.0, Chicago, IL, USA) by one-way ANOVA with Duncan post hoc comparison of means and independent-T-test. Significance was determined at $p < 0.05$. All results were expressed as means ± SD and each experiment was determined in three separate experiments with three replicate.

4.3 Results

4.3.1 Lipids Composition in *Ganoderma lucidum* Extracts

The amount of non-esterified fatty acids (**Figure 7**) in each of the extracts and fractions were 0.078 ± 0.013 (crude extract), 0.049 ± 0.013 (fraction 1), 0.063 ± 0.013 (fraction 2), 0.113 ± 0.013 (fraction 2) and 0.350 ± 0.033 (fraction 4) mEq/L oleic acid

for every milligram of the extracts. Fraction 4 accumulated approximately 4.5 times non-esterified fatty acids compared to the crude extract.

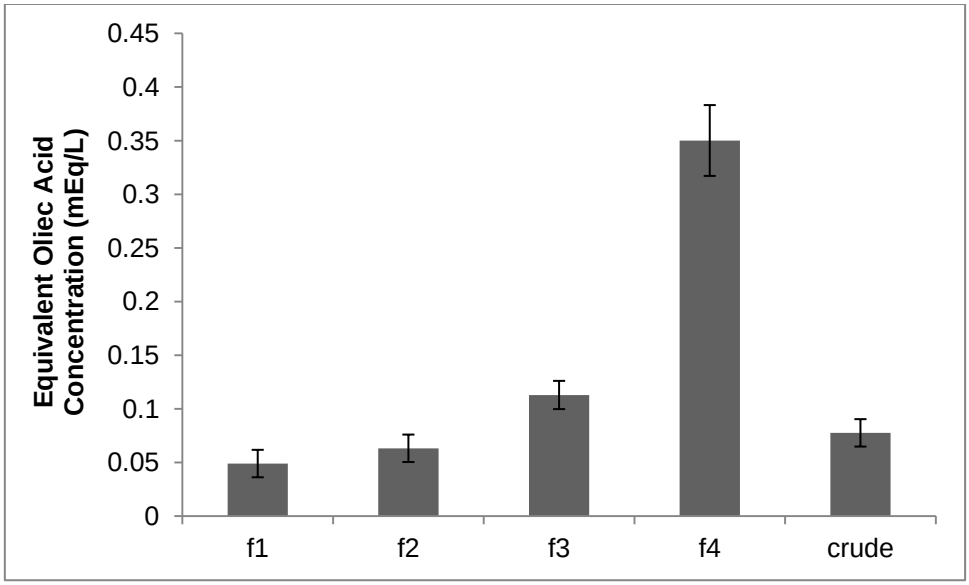


Figure 7. Non-esterified fatty acid content of *Ganoderma lucidum* extracts. Values are expressed as mean ± SD.

The lipid composition of fraction 4 was further analyzed by LC-MS/MS and NMR. Results are shown in **Table 4**. The NMR spectrum was included in Apendix 4. A total of 15 compounds were found in fraction 4 including saturated and unsaturated fatty acids, hydroxyl fatty acids, sterol derivatives, phospholipids and some non-identified compounds. The identified fatty acids in fraction 4 include palmitoleic acid, linoleic acid, palmitic acid, oleic acid and stearic acid, which have been confirmed by matching the ^{13}C NMR data with representative literatures [102]. The MS data shows that the fatty acids tend to form dimmers and lose one molecule of H_2O during ionization. Hydroxyl fatty acids such as methyl hydroxy-oxo-octanoate, hydroxy-hexadecenoic acid, dihydroxy-hydroperoxy-octadecenoic acid, hydroxy-heptadecatrienoic acid and hydroxy-heptadecadienoic acid were also found in fraction 4 which is evidenced by the multiple losses of H_2O in the positive MS fragmentation and some representative

chemical shifts from the NMR spectra such as ^{13}C DEPT δ_{CDCl_3} at 70.5 (*C*HOH), 73.2 (*C*HOH), 40.8(*C*H_2CHOH), 36.9(CHOH*C*H_2). The other compounds were believed to be sterol derivatives with representative chemical shifts of 70.5(CHOH), 12.1(CH_3), 116.3(CH), 119.6(CH), 16.3(CH_3), 21.1(CH_3), 135.5(CH), 17.6(CH_3), 19.7(CH_3), 19.9(CH_3) ppm in ^{13}C DEPT spectra and phospholipids, identified as phophatidylcholine with representative chemical shifts of 70.2, 70.5 (CH sn2), 54.6(N (CH_3)$_3$) ppm from ^{13}C DEPT spectra.

Table 4. Representative MS fragments and chemical shift of ^{13}C DEPT NMR spectra of major compounds in *Ganoderma lucidum* fraction 4 extract.

Retention Time (min)	Compound	Representative MS fragments	Chemical shift in ^{13}C DEPT NMR spectra (ppm)
		fatty acids	
20.2	palmitoleic acid	255.2$[M+H]^-$, 507.6$[2M-H]^-$, 238.2, 220.3, 165.2, 136.7, 93.3	29.1(C6),22.7(C7),29.0(C8),
26.2	linoleic acid	280.2$[M]^-$,559.5$[2M-H]^-$, 262.0$[M- H_2O]^-$	34.2(C2),24.8(C3),29.2-29.7(C4-C7), 27.2(C8), 130.0(C9),128.1(C10),25.6(C11),128.1(C12),130.0(C13), 27.2(C14), 29.3(C15),31.9(C16),22.6(C17),14.1(C18)
28.7	palmitic acid	257.4$[M+2-H]^-$, 512.6$[2M]^-$,245.7	34.2(C2),24.7(C3),29.2-29.7(C4-C13),31.9(C14), 22.7(C15),14.1 (C16)
29.5	oleic acid	282.2$[M]^-$, 563.6$[2M-H]^-$, 263.4$[M-H-H_2O]^-$	34.2(C2),24.8(C3),29.5-29.8(C4-C7),27.2(C8),129.7, 130.0(C9-10),27.2(C11),29.5-29.8(C12-C15),31.9(C16), 22.6(C17),14.1 (C18)
33.3	stearic acid	284.4$[M]^-$,567.6$[2M-H]^-$, 266.6$[M-H-H_2O]^-$, 148.6	34.2(C2), 24.8(C3),29.2-29.7(C4-C15), 31.9(C16),22.7(C17),14.1 (C18)
		hydroxy fatty acids	
3.7	methyl hydroxy-oxo-octanoate	311.9$[M]^+$,294.0,283.9,269.0,228.1,211.2,185.4	70.5 (CHOH), 73.2 (CHOH), 40.8(CH_2CHOH),36.9(CHOHCH_2)
4.5	hydroxy-hexadecenoic acid	270.1$[M]^+$,212.2,184.3,167.4	

9.9	unknown	319.0$[M+H]^+$, 301.0, 283.1, 271.1, 253.3, 204.9,108.8	
11.1	unknown	313.0$[M+H]^+$, 295.0, 277.1, 260.1, 249.8, 234.2, 204.1, 134.7,120.8	
11.8	unknown	333.0$[M+H]^+$, 315.0,307.7, 297.1, 285.1,279.1, 226.3	
12.5	unknown	331.0$[M+H]^+$, 313.1, 295.0, 263.5	
13.7	dihydroxy-hydroperoxy-octadecenoic acid	347.0$[M+H]^+$, 329.0, 311.0, 294.0, 264.2, 213.4	
21.9	hydroxy-heptadecatrienoic acid	281.1$[M+H]^+$,559.6$[2M-H]^+$,264.1 ,246.1, 190.3, 176.4, 162.4,148.6,122.8,111.1,99.1,87.2	
25.5	hydroxy-heptadecadienoic acid	283.1$[M+H]^+$,563.6$[2M-H]^+$,266.1, 248.2, 192.3, 178.4, 164.6,150.7,122.8,97.1	
		sterol derivatives	
29.8	unknown	417.9$[M]^-$,360.0, 380.0, 337.9	70.5(CHOH),12.1(CH_3),116.3(CH),119.6(CH), 16.3(CH_3),21.1(CH_3),135.5(CH),17.6(CH_3),19.7, 19.9(CH_3)
34.5	unknown	531.5$[M+H]^+$,427.2, 413.7,391.8	
39.5	unknown	587.6$[M+H]^+$, 448.7, 285.8	
		phospholipids	
40.9	phosphatidylcholine	817.6$[M-H]^-$, 754.8,256.7, 282.8	70.2,70.5 (CH sn2),54.6($N(CH_3)_3$)
24.3	fatty ester	257.2$[M+H]^+$,168.4,131.7,104.2	
8.2	unknown	325.9$[M+H]^+$, 282.9, 269.9, 252.1, 240.9, 224.1 203.2	

4.3.2 Cytotoxicity of the Extracts and Their Induction of G2/M Arrest

The effects of the crude extract and fraction 4 treatments on Caco-2 cell viability are shown in **Figure 8**. The crude extract inhibits the cell growth of Caco-2 cells in a dose dependent manner when exposed before cell confluency (24 h after seeding) with an LC50 of 0.336 ± 0.071 mg/mL. The LC 50 was determined by the linear equation of y = − 50.599 x + 25.93 (R^2 = 0.9267) obtained by plotting cell viability against log concentrations (graph not shown). However, when treated with the crude extract after cell confluency (4 days after seeding), cells are resistant to the cytotoxic effects of crude extract with cell viability of 63.28 ± 7.81% at a concentration of 1 mg/mL compared to untreated control cells. Fraction 4 showed little effects on cell growth, the LC50 could not be established up to a tested concentration of 1 mg/mL and showing a 63.46 ± 5.51% and 70.61 ± 1.82% viability for undifferentiated and confluent cells respectively.

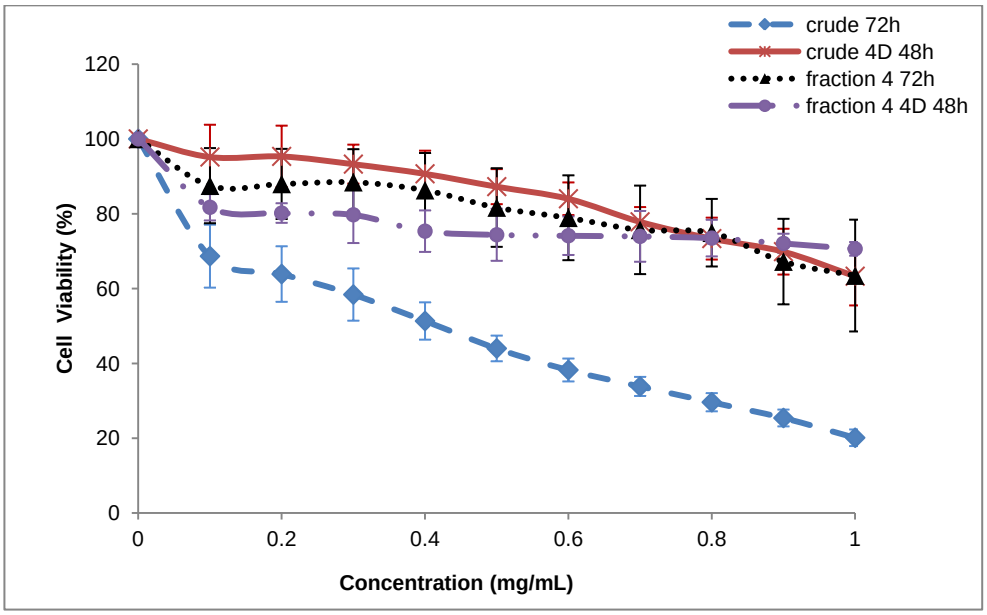


Figure 8. Cell viability of the crude extract and fraction 4 treated Caco-2 cells by MTT assay. Values are expressed as mean ± SD (percentage of untreated control cells). Crude 72 h and fraction 4 72 h cells represent cells that were seeded for 24 h and treated for 72 h; Crude 4D 48 h and fraction 4 4D 48 h, represents cells that were cultured for 4 days before treatments.

The cell cycle analysis is summarized in **Table 5**. Both the crude extract and fraction 4 caused a minor G2/M arrest in cell cycle distribution with 4.78% and 7.25% increase for the crude extract and 6.56% and 5.30 % increase for fraction 4 after 48 and 72 h treatments respectively. However, no significant accumulation was observed in sub-G1, which indicated that neither of the treatment induced DNA fragment in Caco-2 cells.

Table 5. Cell cycle distribution of Caco-2 cells for treatments of crude extract at the LC50 concentration from MTT assay and fraction 4 with a concentration of 0.5 mg/ml. Values are expressed as mean ± SD. An asterisk represents significant differences ($p < 0.05$) in the same phases within the same period compared to control values.

			Sub-G1	G0/G1	S	G2/M
Crude	24 hr	control	3.06±0.89	38.76±1.91	20.84±1.82	36.48±1.89
		sample	2.63±0.46	33.47±1.52*	20.60±1.97	41.63±1.84*
	48 hr	control	2.72±0.48	45.14±1.56	18.96±1.63	30.29±0.83
		sample	3.20±0.88	38.61±1.19*	22.33±1.34*	35.07±0.42*
	72 hr	control	3.28±0.91	49.17±1.71	19.27±1.64	28.11±1.73
		sample	3.16±0.45	41.43±2.78*	20.01±2.42	35.36±2.43*
Fraction 4	24 hr	control	3.25±0.37	47.23±3.77	16.90±1.84	30.60±1.11
		sample	2.47±1.21*	45.76±2.14	16.33±2.17	35.33±5.52*
	48 hr	control	1.55±0.19	42.33±3.55	17.80±2.86	38.30±1.13
		sample	1.49±0.45	39.12±2.37*	14.57±1.98*	44.86±1.64*
	72 hr	control	1.58±0.44	46.04±1.04	15.84±1.77	36.53±1.25
		sample	1.48±0.65	42.94±1.925	13.95±2.28	41.83±0.77*

4.3.3 Induction of Differentiation in Caco-2 Cells by *Ganoderma lucidum* Extracts

The effects of the crude extract and fraction 4 on cell differentiation were determined by measuring the activities of two differentiation enzyme markers, alkaline phosphatase and lactase. Both the crude extract and fraction 4 are able to increase the activity of alkaline phosphatase and lactase compared to the control (**Figure 9**). The alkaline phosphatase activities significantly ($p < 0.05$) increased 129% for both the crude

extract and 189% for fraction 4 treatment compared to untreated control cell. The lactase activities increased significantly ($p < 0.05$) both for treatments of the crude extract (32%) and fraction 4 (113%).

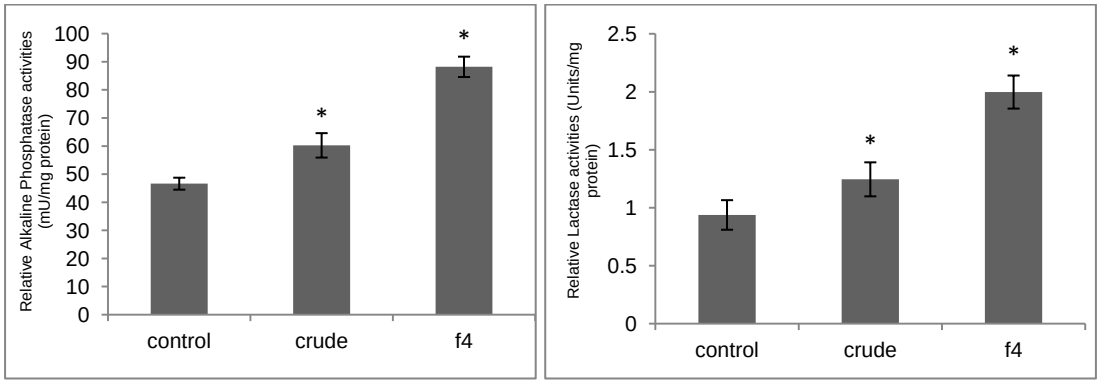


Figure 9. Alkaline phosphatase (left panel) and lactase activities (right panel) in Caco-2 cells with treatment of *Ganoderma lucidum* extracts. Values are expressed as mean ± SD. An asterisk represents significant differences ($p < 0.05$) compared to control values.

4.3.4 Effects of JNK, ERK and PKC Pathway on Alkaline Phosphatase Activity

Effects of JNK, MEK/ERK and PKC inhibitor on alkaline phosphatase activitiy are shown in **Table 6**. Confluent Caco-2 cells treated with one of three different protein kinase inhibitors did not show any significant ($p > 0.05$) differences in alkaline phosphatase compared to control cells without addition of inhibitors. The addition of the selective JNK inhibitor SP600125 failed to significantly ($p > 0.05$) activate the alkaline phosphatase activity by treatments of the crude extract and fraction 4, as shown in **Table 6**. The relative alkaline phosphatase activity reduced 28% and 87% for treatments of the crude extract and fraction 4 respectively after adding SP600125 compared to treatments without inhibitor (e.g. ratio of alkaline phosphatase from treatments to control− ratio of alkaline phosphatase from treatments with inhibitors to control with inhibitors). Similarly, the MEK/ERK-1/2 inhibitor U0126 was able to block the stimulation of alkaline phosphatase activity induced by the crude extract and fraction 4, with reductions of

relative enzyme activity of 25% (crude extract) and 84% (fraction 4) compared to treatments without inhibitor. With addition of the PKC inhibitor Ro31- 8220, the stimulated alkaline phosphatase activity by the crude extract treatment was attenuated significantly ($p < 0.05$) by 21% of relative activity compared to treatment without inhibitor. While treatment of fraction 4 still significantly ($p < 0.05$) stimulated alkaline phosphatase activity (6.88%) compared to control cells with addition of inhibitor in which PKC pathway was blocked by Ro31- 8220. However, the enzyme level was also significantly ($p < 0.05$) reduced (75%) compared to treatment of fraction 4 without PKC inhibitor.

Table 6. Effects of JNK inhibitor SP600125, MEK/ERK inhibitor U0126, PKC inhibitor Ro31-8220 and *Ganoderma lucidum* extracts on alkaline phosphatase activities. Inhibitors were added 30 min prior *Ganoderma lucidum* extracts treatments. Values are expressed as mean ± SD. An asterisk represents significant differences ($p < 0.05$) compared to values of untreated cells with inhibitors alone.

	Relative alkaline phosphatase activities (mUnits/mg protein)		
	MEK/ERK inhibitor (U0126)	JNK inhibitor (SP600125)	PKC inhibitor (Ro31-8220)
Untreated cells + inhibitor	54.04±3.47	46.08±4.30	48.53±4.73
Crude extract + inhibitor	56.16±3.04	46.38±4.63	52.23±4.06
Fraction 4 + inhibitor	56.95±6.50	46.83±4.00	55.41±5.10*

4.4 Discussions

In this chapter, a crude extract and a lipid containing fraction were prepared from *Ganoderma lucidum*. The crude extracted contained a known class of cytotoxic triterpenoids that we previously reported in charptor 3 to induce apoptotic cell death in Caco-2 colon carcinoma cells [103]. We anticipated a reduction in cell viability and an arrest of the cell cycle in Caco-2 cells using a crude extract which contained triterpenoids. These apparent cytotoxic effects were diminished once the cells reached confluency and treated with the crude extract. Utilizing flash chromatograph four fractions were created

by elution with different solvent ratios. Each fraction was separately tested for viability as reported in chapter 3. Fraction 4 did not have any effect on cell growth or apoptosis but changed the morphology of Caco-2 cells which appeared similar to Caco-2 differentiation. We reported herein that fraction 4 contained 4.5 times the non-esterified fatty acid content compared to the crude extract and had no measureable triterpenoid content. It was noticed that the non-estrified fatty acid content in all extracts was relatively low and below 1 mEq/L per milligram, which could be due to the low solubility of fatty acids in aqueous solusion tested. LC-MS and NMR analysis showed that fraction 4 contained long chain saturated and unsaturated fatty acids, hydroxyl fatty acids, sterol derivatives and phospholipids. The saturated and unsaturated fatty acid profile has been confirmed by MS and NMR analysis and consistent to those reported in *Ganoderma lucidum* extracts [8;104]. Part of the hydroxyl fatty acids and phospholipids were identified while some of the sterol derivatives remain unidentified.

Ganoderma lucidum has been reported to possess multiple pharmaceutical functions in chemoprevention as stated in chapter 2 [7;45;52;54]. Treatments using the crude extract and fraction 4 both significantly ($p < 0.05$) increased enzymes activities of alkaline phosphatase and lactase, which are the two well accepted markers of colon epithelial cell differentiation [93;105;106]. Moreover, fraction 4 increased alkaline phosphatase activity 60% and lactase activity 81% more than the crude extract under the same concentration (0.5 mg/mL). Therefore, the induction of Caco-2 differentiation is stronger for the lipid concentrated fraction of *Ganoderma lucidum*. Similarly, dietary polyunsaturated fatty acids have been reported to the influence breast cancer progression in MCF-7 human breast cancer cells and induction of differentiation served as the main pathway for the growth inhibitory effects [107]. Regulation of the balance between proliferation and differentiation was thought to be important in the development of

neoplastic transformation of colonic epithelium [108]. Cells undergo differentiation were thought to lose the capability of proliferation and have shorter life span, which serve as an effective elimination process for damaged cells to avoid neoplastic transformation [76]. The use of non-cytotoxic agents including differentiation agents to treat colon cancer has been suggested to help restore normal growth control in carcinoma cells [94]. It has also been reported that induction of differentiation was associated with suppression of malignant phenotype in colon carcinoma [109;110]. Thus, induction of differentiation by *Ganoderma lucidum* extracts suggests that lipid component may play a role in the regulation of differentiation of cultured colonic cells.

Short chain fatty acids, produced by colonic fermentation, such as butyrate, induced colon epithelial differentiation and apoptosis [93]. However, the effects of other lipids on colon epithelial differentiation have not been adequately reported in the literature. Unlike the short chain fatty acids, the lipids enriched fraction (fraction 4) from *Ganoderma lucidum* neither exhibited cytotoxicity to Caco-2 cells nor induced apoptosis even at a high concentration (1 mg/mL). Although the crude extract inhibited cell growth in undifferentiated Caco-2 cells, the inhibitory effects diminished after cells reaching confluency. This could be a result of rapid metabolism of toxic substance in the extract when cells undergo differentiation after reaching confluency [111].

Regulation of the cell cycle has been reported in colonic epithelial cells differentiation and sodium butyrate induced differentiation was thought to involve acetylcholinesterase mediated G2/M cell cycle arrest [112]. Cell cycle arrest at G1/S or G2/M phases was also believed to correlate with differentiation in Caco-2 cells [113]. A G1 arrest was associated with Caco-2/15 cells differentiation [114]. In our study, a G2/M cell cycle arrest was observed from both treatments. Fraction 4 increased the cells in maximum at the G2/M phase of 7.25% at 48 h while the crude extract showed an increase

of 4.78%. The crude extract showed a maximum increase of 6.56% at 72 h compared to 5.30 % for fraction 4. The different responses of cell cycle arrest for the crude extract and fraction 4 may due to the triterpenoid components in the crude extract while Fraction 4 does not contain any measurable triterpenoids. Triterpenoid rich fraction has been reported to cause a G2/M arrest in Caco-2 cells [115]. It is likely that the lipid component of fraction is arresting the cell in a different manner than triterpenoids. Similarly, alkaline phosphatase and lactase activities were much greater from treatment with fraction 4 at 48 h compared to the crude extract in confluent cells. However, whether the cell cycle arrest play a role in *Ganoderma lucidum* induced- Caco-2 differentiation is not yet clear and need further study.

Multiple signal transduction pathways have been reported to be responsible for mediation of differentiation process in Caco-2 cells. The effects of the addition of three protein kinase inhibitors were examined. Stimulation of alkaline phosphatase activity during differentiation was inhibited by adding specific inhibitors of JNK (SP600125), MEK/ERK (U0126) and PKC (Ro-31-8220). Alkaline phosphatase activity did not increase by treatment with either the crude or fraction 4 combined with the inhibitors of JNK and MEK/ERK pathways, the enzymatic levels were unchanged compared to untreated cell plus those two inhibitors. Therefore alkaline phosphatase activity was completely inhibited by JNK and MEK/ERK inhibitors, which suggested these pathways were involved in the *Ganoderma lucidum* induced differentiation reported herein. Activation of JNK has been reported to contribute to the spontaneous differentiation of Caco-2 and sodium butyrate induced-differentiation [93;101]. Activation of JNK was also reported to correlated to differentiation induced by 1,25-Dihydroxyvitamin D_3 , which was a potential chemo-preventive agent for colon cancer [116]. However, in spontaneous differentiating Caco-2 cells, MEK/ERK was reported to be inhibited [101]. This is

contrary to our finding that MEK/ERK was activated in *Ganoderma lucidum* induced differentiation. The MEK/ERK inhibitor U0126 used in this study has been reported to have the potential to antagonize AP-1 by noncompetitive inhibition, making it potential inhibitor of AP-1 [117]. The inhibition of activator protein-1 rather than ERK is very likely to result in suppression of differentiation and reduced the *Ganoderma lucidum* stimulate-alkaline phosphatase activity. It is plausible that activator protein -1 could also be involved *Ganoderma lucidum* induced-differentiation. Stimulation of the AP-1 was found to be associated with 1,25-Dihydroxyvitamin D_3 induced- differentiation in Caco-2 cells [116].

The crude extract combined with the PKC inhibitor Ro-31-8220 also resulted in a complete inhibition of alkaline phosphatase activity, which was evident by a similar level of alkaline phosphatase activity compared to untreated cell plus Ro-31-8220. However, fraction 4 combined with the PKC inhibitor resulted in a 6.88% increase in activity, indicating a lack of full inhibition of alkaline phosphatase activity from PKC inhibitor combined with fraction 4 treatment. Therefore, PKC may play a role in the observed differentiation but its activation is probably indirectly affected by *Ganoderma lucidum*. Differentiation induced by sodium butyrate and 1,25-Dihydroxyvitamin D_3 were also reported to be mediated through activation of PKC in Caco-2 cells [93;116].

4.5 Conclusions

An ethanol extract and its lipid fraction from *Ganoderma lucidum* were found to be non-cyctotoxic and both induce differentiation in Caco-2 cells with an increase in alkaline phosphatase and lactase activity compared to untreated control cells. The differentiation induced by *Ganoderma lucidum* lipids was possibly mediated through activation of JNK and ERK pathways. The lipid composition of *Ganoderma lucidum* induced differentiation in Caco-2 and these effects differed from those of short chain fatty acids.

CHAPTER 5 EXTRACTION OPTIMIZATION AND ISOLATION OF TRITERPENOIDS FROM GANODERMA LUCIDUM AND THEIR CYTOTOXICITIES TO CULTURED HUMAN CARCINOMA CELLS

Preface

Selected portion of Chapter 5 are ready to be submitted as:

Ruan, W.; Lim H.H.A.; Lau G.H.; Popovich, D. G. Extraction optimization and isolation of triterpenoids from *Ganoderma lucidum* and their cytotoxicities to cultured human carcinoma cells. *xxx*. **2013**.

5.1 Introduction

As stated in chapter 2, *Ganoderma lucidum* is a popular medicinal mushroom used as a folk remedy in Asia since ancient time due to its diverse health-promoting properties [118;119]. Of special interest is the reported chemo-preventative properties of both the polysaccharides and triterpenoids isolated from *Ganoderma lucidum* [7;119]. In chapter 3, two triterpenoid extracts of *Ganoderma lucidum* (fraction 2 and 3) inhibited cell growth in Caco-2 human colon carcinoma cells and one of them with less polarity (fraction 3) were able to induce apoptotic cell death [120].

Extraction conditions can alter the biochemical profiles of extracts, which subsequently influence their bioactive potential. A maximum extraction efficiency of target components can be obtained by optimization of the extraction conditions. Response surface methodology (RSM) is a well-accepted approach for optimization extractions and this method requires fewer experimental trials to evaluate influence of multiple parameters such as extraction time, extraction temperature and solvent ratio and their interactions [121;122]. It has been reported that the extraction efficiency of polysaccharides from *Ganoderma lucidum* can increase from 42% to 75% using RSM guided ultrasound-assisted extraction [123]. However, the effects of extraction conditions

on extraction efficiency of triterpenoids from *Ganoderma lucidum* have not been reported. The objective of this chapter is to evaluate and optimize the extraction conditions for *Ganoderma lucidum* triterpenoids, isolate individual triterpenoids and investigate their bioactivities in three cultured human cancer cell lines.

5.2 Materials and Methods

5.2.1 Extraction Process

Approximately 2 g of *Ganoderma lucidum* powder purchased locally (slice of *Ganoderma lucidum*, *chizhi*, origin from China) was weighted and immersed into 30 mL of extraction solvent. The solvent composition, extraction time and extraction temperature were varied according to the experimental design (see below). After the extraction, samples were filtered under vacuum and solvent was evaporated. The aqueous solution of each sample was then lyophilized, weighted and stored at -20 ºC until further analysis. Yields of total extract and ganoderic acid H was used as indexes of extraction efficiency and calculated as follow:

$$\text{Yield of total extract } (Y_1)(\text{mg/g powder}) = \frac{W_2\,(\text{g}) \times C_1\,(\text{mg/g}\,W_2)}{W_1\,(\text{g})}$$

$$\text{Yield of ganoderic acid H } (Y_2)\,(\text{mg/g powder}) = \frac{W_2(\text{g}) \times C_2(\text{mg/g}\,W_2)}{W_1\,(\text{g})}$$

Where W_1 is the accurate weight of the *Ganoderma lucidum* powder used for extraction, W_2 is the weight of total extract powder after lyophilization. C_1 represents the equivalent ganoderic acid H concentration of the total extract in 1 g substance powder after lyophilization and C_2 is the ganoderic acid H concentration per gram extract powder obtained from the HPLC analysis.

5.2.2 Experimental Design

The Box–Behnken factorial design (BBD Design Expert software, Trial Version 8.0, Stat Ease Inc., Minneapolis, MN, USA) was applied to determine the experimental conditions which combined three independent variables including extraction time (X_1), percentage of ethanol in water (X_2) and extraction temperature(X_3). The range for each variable was selected based on a preliminary experiment. The design consisted of 15 experiments (**Table 7**) with three replicates at center points for an experimental error sum of squares evaluation and all experiments were conducted in randomized order. Data were analyzed by a quadratic polynomial model, which expresses the response as a function of the independent variables as follow:

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum\sum_{i<j} \beta_{ij} X_i Y_j$$

Where Y is the response measured, $\beta_0, \beta_j, \beta_{jj}$ and β_{ij} represent the regression coefficients of intercept, linearity, quadrate and interaction respectively. X_i and $Y_j$are the levels of independent variables.

5.2.3 Determination of Triterpenoids by HPLC

Analytical HPLC was conducted on a Waters (Milford, MA, USA) 2695 Separation system coupled with Waters 2996 Photodiode Array (PDA) detector. A reverse phase Agilent Prep C-18 Scalar column (4.6 mm × 250 mm, 10 μm particle size) (Agilent Technologies Inc., Santa Clara, CA, USA) was used with a total flow rate of 1 mg/mL. The mobile phase consisted of 1% acetic acid (A) and acetonitrile (B). Gradient conditions were applied as followed: 0 - 5 min, 5% B - 35% B; 5 - 25 min, 35% - 45% B; 25 - 35 min, 45% B - 60% B; 35 - 45 min, 60% B - 75% B; 45 - 50 min, 75% B - 95% B;

50 - 60 min, 95% B - 100% B; 60 - 70 min, 100% B. The detection wavelength was set at 254 nm.

An external standard of ganoderic acid H (Planta Analytica, Irvine, CA, USA) with five concentrations ranged from 10 to 100 μg /mL was used for relative quantification. Linearity of the calibration curve was determined by plotting concentrations against peak areas.

Each sample extracted using the BBD model was dissolved in 50% methanol to make 1 mg/mL solution and filtered through 0.45 μm filter (Millex GP, Millipore, Billerica, MA, USA) and stored at 4 ºC before use. 10 μL of sample was injected for HPLC analysis and the component concentrations in samples were calculated using the linear equation of the standard curve.

5.2.4 Isolation of Individual Triterpenoid

Crude extraction was carried out under the optimal condition obtained from RSM. The crude extract was further separated into four fractions using flash chromatography as described in chapter 3 [124]. The triterpenoid-enriched fraction (fraction 2) was separated by semi-preparative HPLC using a Waters system (Milford, MA, USA) with Waters 515 HPLC pump, Waters 717 plus autosampler and Waters 2996 PDA detector. A Warers XTerra Prep RP-18 column (7.8 mm × 100 mm, 5 μm particle size) (Milford, MA, USA) was loaded and a flow rate of 2 ml/min was applied. A mobile phase of deionized water (A) and acetonitrile (B) was used with a gradient elution of 35% B at 0 -7 min, 35 - 40% B at 7 - 8 min and 40 - 44% B at 8 - 15 min. Samples were prepared in 50% ethanol with a concentration of 10 mg/mL and 200 μL was injected for each isolation. A total of 23 fractions were collected according to the UV absorbance and each fraction was evaporated under vacuum to remove solvents and lyophilized.

5.2.5 ESI-MS Detection and NMR Determination

A Bruker amaZonX IonTrap Mass Spectrometer (Bruker Daltonics Inc., Billerica, MA, USA) was used for molecular weight determination. The ESI-MS conditions were set as follows: dry gas flow rate at 8.0 mL/min, capillary voltage at 4.5 kV, end plate offset at -500 V, capillary temperature at 250 ^{o}C, flow rate at 0.2 mL/min and injection volume of 5 μL. Both positive and negative ionization were used and scanning mass spectra was ranged from 70 to 2000 m/z.

Samples (2-3 mg) were dissolved in 600 μL deuterated chloroform for NMR analysis, which was conducted on the Bruker AC300 or AMX500 MHz Nuclear Magnetic Resonance Spectrometers (Bruker BioSpin GmbH, Rheinstetten, Germany). ^{1}H, ^{13}C and DEPT ^{13}C NMR spectra were measured and chemical shifts were expressed in terms of δ (ppm).

5.2.6 Cell Culture

The Hep G2 cells, Caco-2 cells and HeLa cells derived from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM (Caisson Laboratories, Inc.) with supplement of 10% fetal bovine serum (Hyclone UK Ltd.) and 100 units/mL of penicillin /streptomycin (Gibco, Invitrogen). The cells were maintained at a concentration between 2×10^5 and 1×10^6 cells/mL and subcultured every 3 - 4 days using 0.25% (w/v) trypsin - 0.53 mM ethylenediaminetetraacetic acid solution (Gibco, Invitrogen) for a total replacement of media. Viable cells were assessed by staining cells with 0.04% trypan blue exclusion dye (MP Biomedicals) and counted in a hemocytometer.

5.2.7 MTT Assay

Cytotoxicity of *Ganoderma lucidum* was evaluated by MTT assay. A seeding concentration of 2.0×10^4, 2.5×10^4 and 1.5×10^4 cells/mL for Caco-2, Hep G2 and

HeLa cells respectively were used in 96-well plates. Cells were allowed to attached for 24 h and treated with each individual triterpenoid (described below) at concentrations between 0.1 and 0.7 mg/mL for 72 h. Untreated cells acted as controls. Cell viability was measured as previously described in chapter 3 [125].

5.2.8 Statistical Analysis

The data from RSM were analyzed using Design Expert software (Trial Version 8.0, Stat Ease Inc., Minneapolis, MN, USA) by F-test and ANOVA. Significance was determined at $p < 0.05$. Results of the MTT assay were expressed as means ± SD. ESI-MS detection and MTT assay were conducted in three separate experiments with three replicate.

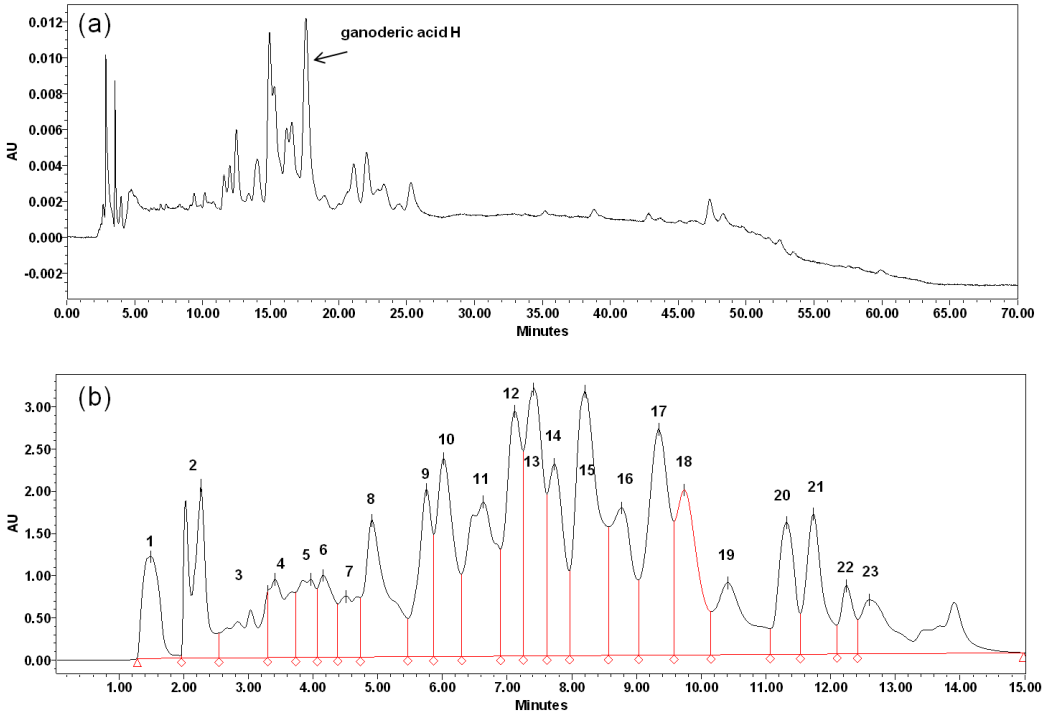
5.3 Results and Discussions

5.3.1 Optimization of Extraction Conditions using RSM

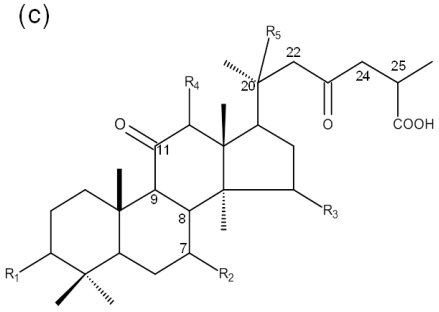
The representative HPLC profile of crude extraction of *Ganoderma lucidum* is shown in **Figure 10(a)**. To quantify the relative concentrations of the extracts, a standard calibration curve of ganoderic acid H is obtained by plotting peak areas against concentrations and linear equations of $y = 6000000\ x - 10414$ ($R^2 = 0.9991$) is determined (graph not shown). The yields of ganoderic acid H (Y_2) and total extract (Y_1) from all *Ganoderma lucidum* extractions are determined and shown in **Table 7** in terms of equivalent ganoderic acid H content per gram powder of *Ganoderma lucidum*. The quadratic polynomial model is simulated based on the experimental data and equations of yields of total substance and ganoderic acid H with linear, square and interaction coefficients are shown as follow:

$$Y_1 = 10.4524 - 3.847660X_1 - 0.10129X_2 + 0.28177X_3 + 0.012779X_1X_2$$
$$- 0.00604503X_1X_3 + 0.00127011X_2X_3 + 0.49981X_1^2$$
$$- 0.0000554307X_2^2 - 0.00305822\,X_3^2$$

$$Y_2 = 1.18317 - 0.35424X_1 - 0.00748798X_2 + 0.038435X_3 + 0.0018X_1X_2$$
$$- 0.00196658X_1X_3 + 0.00018625X_2X_3 + 0.049545X_1^2$$
$$- 0.0000461535X_2^2 - 0.000376988\,X_3^2$$



(c)



compound	R1	R2	R3	R4	R5	double bond
ganoderic acid I	βOH	βOH	O	H	αOH	8,9;
ganoderic acid G	βOH	βOH	O	βOH	H	8,9;
ganoderic acid B	βOH	βOH	O	H	H	8,9;
ganoderic acid A	O	βOH	αOH	H	H	8.9;
ganoderic acid H	βOH	O	O	βOAc	H	8,9;
ganoderenic acid D	O	βOH	O	H	-	8,9; 20,22
ganoderic acid D	O	βOH	O	H	H	8,9;

Figure 10. (a) Representative HPLC chromatograph of *Ganoderma lucidum* extract under one exraction condition with BBD. Ganoderic acid H was calibrated using an external standard. **(b)** HPLC profile of triterpenoid enriched fraction (fraction 2) separated from *Ganoderma lucidum* extract with optimal extraction condition by semi-preparative HPLC isolation. Peak numbers refer to the collected fractions. 8, ganoderic acid I; 12, ganoderic acid G; 14, ganoderic acid B; 17, ganoderic acid A; 18, ganoderic acid H; 20, ganoderenic acid D;

21, ganoderic acid D. **(c)** Chemical structures of identified triterpenoids from *Ganoderma lucidum* triterpenoid enriched fraction (fraction 2).

Table 7. Experimental design of *Ganoderma lucidum* extraction and responses to extract parameters.

Run	Extraction time (h)	Ratio of ethanol in water (%)	Extraction temperature (^{o}C)	Yields (mg/g powder)			
				Total extract		Ganoderic acid H	
	X_1	X_2	X_3	Experimental (Y_1)	Predicted	Experimental (Y_2)	Predicted
1	2.0 (-1)	100.0 (1)	50.0 (0)	9.057	8.818	1.57	1.54
2	2.0 (-1)	50.0 (0)	25.0 (-1)	7.265	7.249	1.26	1.22
3	4.0 (0)	50.0 (0)	50.0 (0)	9.187	8.821	1.48	1.48
4	4.0 (0)	100.0 (1)	25.0 (-1)	4.937	5.191	0.99	1.06
5	6.0 (1)	50.0 (0)	75.0 (1)	9.950	9.963	1.43	1.47
6	4.0 (0)	50.0 (0)	50.0 (0)	8.316	8.821	1.50	1.48
7	6.0 (1)	100.0 (1)	50.0 (0)	13.226	13.324	2.09	2.03
8	4.0 (0)	50.0 (0)	50.0 (0)	8.960	8.821	1.46	1.48
9	6.0 (1)	50.0 (0)	25.0 (-1)	10.157	9.804	1.57	1.55
10	6.0 (1)	0.00 (-1)	50.0 (0)	9.751	9.989	1.20	1.23
11	2.0 (-1)	50.0 (0)	75.0 (1)	8.265	8.618	1.51	1.53
12	4.0 (0)	0.0 (-1)	25.0 (-1)	7.473	7.587	1.11	1.09
13	2.0 (-1)	0.0 (-1)	50.0 (0)	10.694	10.595	1.40	1.46
14	4.0 (0)	100.0 (1)	75.0 (1)	9.245	9.130	1.62	1.64
15	4.0 (0)	0.0 (-1)	75.0 (1)	5.430	5.175	0.80	0.73

* refers to mg ganoderic acid H equivalents/g powder.

The significance of each coefficient are determined by the F-value and p-value as listed in **Table 8**. Since a greater F -value and smaller p-value indicated a more significant variable affecting the extraction [126], the most significant factors influencing the total extract were the linear term of extraction time (X_1), interaction of extraction time and ethanol ratio (X_1X_2), interaction of ethanol ratio and extraction temperature (X_2X_3) ,the quadratic terms of extraction time (X_1^2) and extraction temperature (X_3^2). The most significant factors that affected the yield of ganoderic acid H included the linear terms of extraction time (X_1) and ethanol ratio (X_2), interaction of extraction time and

ethanol ratio (X_1X_2), interaction of extraction time and ethanol ratio (X_1X_3), interaction of ethanol ratio and extraction temperature (X_2X_3), the quadratic terms of extraction time (X_1^2), ethanol ratio (X_2^2) and extraction temperature (X_3^2).

Table 8. Regression coefficients of predicted quadratic polynomial models for the yields of total extract and ganoderic acid H. X_1refers to extraction time; X_2refers to the ethanol ratio; X_3refers to the extraction temperature.

Source	Sum of Squares		df	Mean Square		F Value		p-value (Prob > F)	
	total extract	ganoderic acid H		total extract	ganoderic acid H	total extract	ganoderic acid H	total extract	ganoderic acid H
X_1	7.61	0.037	1	7.61	0.037	40.2	8.33	0.0014	0.0344
X_2	1.21	0.39	1	1.21	0.39	6.42	88.62	0.0523	0.0002
X_3	1.17	0.024	1	1.17	0.024	6.17	5.43	0.0556	0.0672
$X_1 X_2$	6.53	0.13	1	6.53	0.13	34.52	29.54	0.002	0.0029
$X_1 X_3$	0.37	0.039	1	0.37	0.039	1.93	8.81	0.2233	0.0312
$X_2 X_3$	10.08	0.22	1	10.08	0.22	53.28	49.42	0.0008	0.0009
X_1^2	14.76	0.15	1	14.76	0.15	77.99	33.05	0.0003	0.0022
X_2^2	0.071	0.049	1	0.071	0.049	0.37	11.2	0.5672	0.0204
X_3^2	13.49	0.2	1	13.49	0.2	71.28	46.72	0.0004	0.001
Model	57.64	1.26	9	6.4	0.14	33.85	31.92	0.0006	0.0007
Pure Error	0.41	0.000712	2	0.2	0.000356				
Total	58.59	1.28	14						

ANOVA for the fitted quadratic polynomial models for the extraction are shown in **Table 9**. The coefficient of determination (R^2) are 0.9839 and 0.9829 for the response of total triterpenoids and ganoderic acid H respectively, and large R^2 is indicative that the models adequately represent the experimental data [127]. Thus, the predicted values (**Table 7**) of the amount of total extract and ganoderic acid H obtained from the regression equations were achieved and were similar to experimental values.

Table 9. ANOVA for the fitted quadratic polynomial model of extraction of total extract and ganoderic acid H.

	Total extract	Ganoderic acid H
Mean	8.79	1.4
Std. Dev.	0.44	0.066
R-Squared	0.9839	0.9829
Adj R-Squared	0.9548	0.9521
C.V. %	4.95	4.73

The 3-D response surface plots of yields of total extract and ganoderic acid H was obtained by plotting the response against any two variables while the third variable was fixed constant at its respective zero level and is shown in **Figure 11**. When the extraction was conducted at a medium extraction temperature (50^{o}C, **Figure 11 (a)** and **(d)**), high ethanol concentration (100% ethanol) system with long extraction time (6 h) resulted in a maximum yield of both total extract and ganoderic acid H. The influence of ethanol concentrations in long time extraction (6 h) and effects of extraction time on the yields of total extract and ganoderic acid H were also similar. However, medium extraction time (4 h) seemed to have more negative effects on yield of total extract amount than that of ganoderic acids H. In addition, in a short extraction time (2 h), total extract amount inversely correlated to the ethanol concentration while the yield of ganoderic acid H increased with an accelerated ethanol concentration. When experiments were conducted in a solvent system with 50% ethanol (**Figure 11 (b)** and **(e)**), higher yields of both total extract and ganoderic acid H were observed in a medium extraction temperature with either short or long extraction time. Medium extraction time seemed to reduce the yields of both total extract and ganoderic acid H. Compared to the yield of ganoderic acid H, yield of total extract was more sensitive to the change of extraction time and temperate evidenced by more changeful curves. When 4 h extractions were conducted (**Figure 2 (c)** and **(f)**), extraction temperatures exhibited similar effects on the yields of total extract and ganoderic acid H. The yields of both total extract and ganoderic acid were positively influenced by the temperature in 100% ethanol system while higher yields were obtained

in a medium extraction temperature in a water system. Ethanol concentrations had a positive effect on yields of both total extract and ganoderic acid H in a high extraction temperature environment (75 ^{o}C) while in a low extraction temperature (25 ^{o}C), ethanol concentrations negatively affected the yield of total extract and a medium ethanol concentration generated higher yield of ganoderic acid H. The optimal extraction parameters were predicted to be 100.00% ethanol at 60.22 °C for 6.00 h and 98.59% ethanol at 65.15 °C for 5.99 h for extraction of ganoderic acid H and total extract respectively. The variations of effects of extraction parameters on the amount of total extract and ganoderic acid H are likely a result of structural difference between the total extract and ganoderic acid H. It is possible that the response model of ganoderic acid H represents the corresponding model of triterpenoids as they share relatively similar structures as ganoderic acid H while the response model of total extract represents a combination model of polysaccharides, phenolic acids and triterpenoids, which may exhibit different properties in polarity and heat stability. In order to obtain efficient extraction of triterpenoids, the optimal extraction parameters for ganoderic acid H extraction was used for extraction of triterpenoids from *Ganoderma lucidum*.

The extraction solvent discussed in this chapter was limited to ethanol so that data present in this chapter is comparable to reported yields of triterpenoids from single factor and orthogonal optimizations, which also used ethanol as extraction solvent [71]. In addition, ethanol is considered relatively non-toxic solvent for herbal extraction compared to other organic solvents such as hexane and chrolofrom. It was noticed that repeated extractions were able to increase the extraction efficiency as shown in Appendix 3. However, in order to reduce the workload during modeling extraction conditions, repeated extractions were not included in the experimental design using response surface methodology. Instead, extractions of triterpenoids under optimal extraction condition

obtained from response surface methodology were repeated three times to mixinize the extraction efficiency when large-scale extractions were conducted to obtain individual triterpenoids in subsequent experiments in this chapter and chapter 6 and 7.

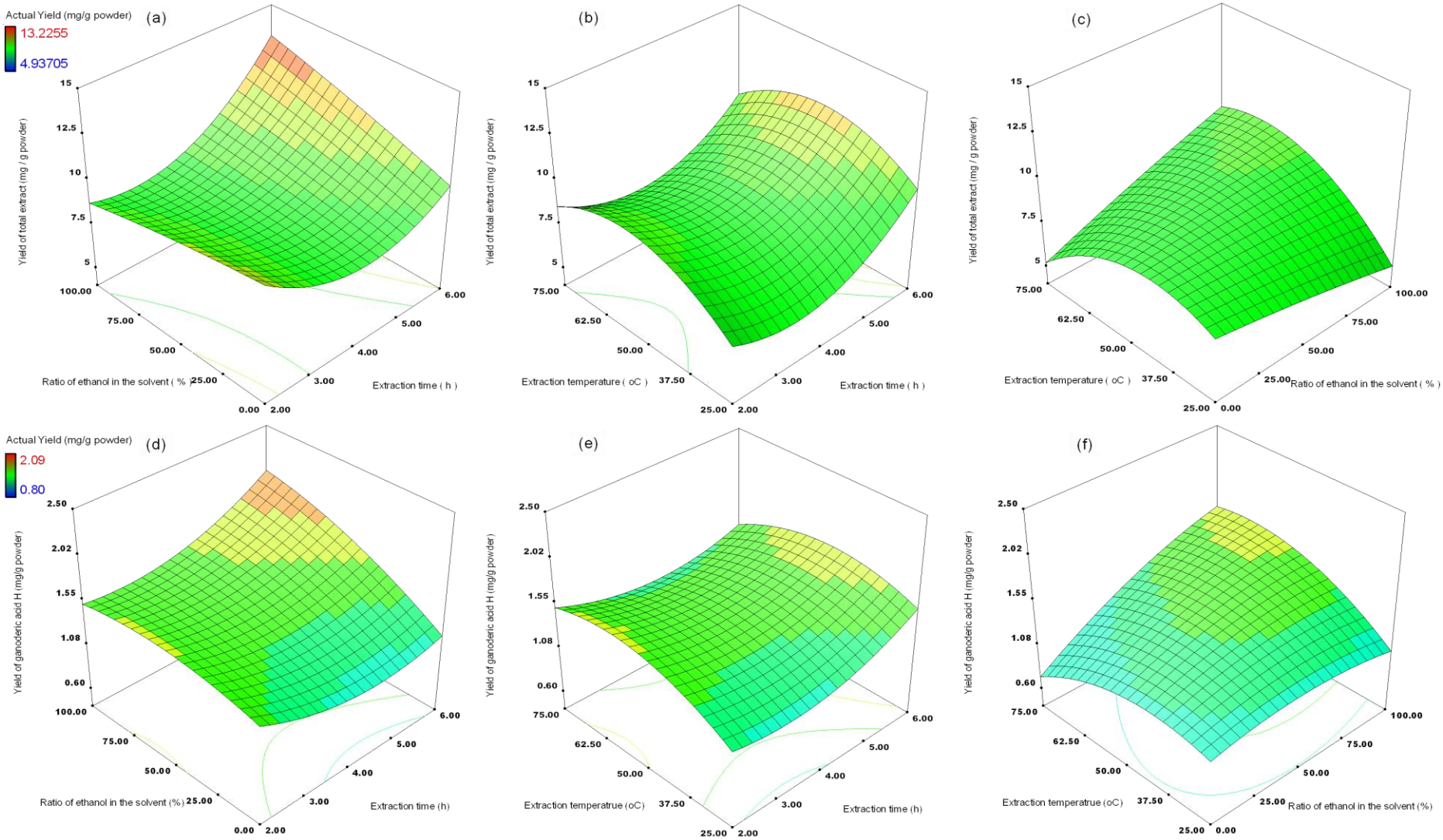

Actual Yield (mg/g powder)
13.2255
4.93705
(a)
Yield of total extract (mg / g powder)
15
12.5
10
7.5
5
100.00
75.00
50.00
25.00
0.00
Ratio of ethanol in the solvent (%)
2.00
3.00
4.00
5.00
6.00
Extraction time (h)
(b)
Yield of total extract (mg / g powder)
15
12.5
10
7.5
5
75.00
62.50
50.00
37.50
25.00
Extraction temperature (oC)
2.00
3.00
4.00
5.00
6.00
Extraction time (h)
(c)
Yield of total extract (mg / g powder)
15
12.5
10
7.5
5
75.00
62.50
50.00
37.50
25.00
Extraction temperature (oC)
0.00
25.00
50.00
75.00
100.00
Ratio of ethanol in the solvent (%)
Actual Yield (mg/g powder)
2.09
0.80
(d)
Yield of ganoderic acid H (mg/g powder)
2.50
2.02
1.55
1.08
0.60
100.00
75.00
50.00
25.00
0.00
Ratio of ethanol in the solvent (%)
2.00
3.00
4.00
5.00
6.00
Extraction time (h)
(e)
Yield of ganoderic acid H (mg/g powder)
2.50
2.02
1.55
1.08
0.60
75.00
62.50
50.00
37.50
25.00
Extraction temperatrue (oC)
2.00
3.00
4.00
5.00
6.00
Extraction time (h)
(f)
Yield of ganoderic acid H (mg/g powder)
2.50
2.02
1.55
1.08
0.60
75.00
62.50
50.00
37.50
25.00
Extraction temperatrue (oC)
0.00
25.00
50.00
75.00
100.00
Ratio of ethanol in the solvent (%)

Figure 11. (a) Response surface plot of total extract (Y_1) as a function of extraction time (X_1) and ethanol concentration (X_2) at an extraction temperature of 50 °C **(b)** Response surface plot of total extract (Y_1) as a function of extraction time (X_1) and extraction temperature (X_3) in a solvent of 50% ethanol **(c)** Response surface plot of total extract (Y_1) as a function of ethanol concentration (X_2) and extraction temperature (X_3) in a 4 h extraction **(d)** Response surface plot of ganoderic acid H (Y_2) as a function of extraction time (X_1) and ethanol concentration (X_2) at an extraction temperature of 50 °C **(e)** Response surface plot of ganoderic acid H (Y_2) as a function of extraction time (X_1) and extraction temperature (X_3) in a solvent of 50% ethanol **(f)** Response surface plot of ganoderic acid H (Y_2) as a function of ethanol concentration (X_2) and extraction temperature (X_3) in a 4 h extraction.

5.3.2 Isolation and Identification of Triterpenoids form *Ganoderma lucidum*

The HPLC profile of fraction 2 obtained by flash chromatography was shown in **Figure 10 (b)**, which was then used to isolate individual triterpenoids. A total of 23 fractions were collected according to the HPLC profile and 7 triterpenoids were identified by ESI-MS and NMR analysis by comparing with the reported literature (NMR spectrums are included in Apendix 5) [32;65;77;128-130]. The molecular weight and structural details are:

Ganoderic acid I ESI-MS m/z: 531 $[M-H]^-$, 1063$[2M-H]^-$,513,469,451, 303, 265. ^{1}H NMR δ_{CDCl_3}: 3.21 (1H, dd, J=6.0, 11.2Hz, 3-H), 0.87 (3H, s, 5-H), 4.79 (1H, t, J=8.4Hz, 7-H), 1.14 (3H, s, 18-H), 1.22 (3H, s, 19-H), 1.39 (3H, s, 21-H), 0.85 (3H, s, 31-H), 1.34 (3H, s, 32-H). ^{13}C NMR δ_{CDCl_3}: 34.9, 27.7, 78.3, 38.8, 49.2, 26.6, 66.9, 156.7, 142.5, 38.7, 198.0, 50.6, 45.6, 59.7, 217.7, 36.1, 49.1, 19.0, 18.4, 73.0, 26.6, 52.6, 210.4, 47.5, 34.2, 175.2, 16.9, 28.2, 15.4, 24.8.

Ganoderic acid G ESI-MS m/z: 531 $[M-H]^-$, 1063$[2M-H]^-$,513,469, 319, 303, 265. ^{1}H NMR δ_{CDCl_3}: 3.21 (1H, dd, J=7.6, 8.8Hz, 3-H), 4.77 (1H, t, J=8.4Hz, 7-H), 4.36 (1H, s, 12-H), 0.79 (3H, s, 18-H), 1.30 (3H, s, 19-H), 1.13 (3H, d, J=6.4Hz, 21-H), 1.21 (3H, d, J=8.0Hz, 27-H), 1.03 (3H, s, 30-H), 0.87 (3H, s, 31-H), 1.44 (3H, s, 32-H). ^{13}C NMR δ_{CDCl_3}: 34.3, 27.5, 78.3, 38.6, 49.1, 26.7, 66.2, 157.3, 141.9, 38.3, 199.3, 77.8, 51.9, 60.3,

216.8, 38.2, 45.7, 11.9, 18.8, 28.7, 21.3, 48.3, 8.2, 46.2, 34.5, 176.1, 16.9, 28.1, 15.3, 23.0.

Ganoderic acid B ESI-MS m/z: 515 $[M-H]^-$, 1031$[2M-H]^-$, 497, 453 , 303, 287, 249. ^{1}H NMR δ_{CDCl_3}: 3.21 (1H, dd, J=6.0, 11.2Hz, 3-H), 0.85 (3H, s,5-H), 4.79(1H, t, J=8.4Hz, 7-H), 1.00 (3H, s, 18-H), 1.21 (3H, s, 19-H), 0.98 (3H, d, J=6.0Hz, 21-H), 1.24 (3H, d, J=6.4Hz, 26-H), 1.03 (3H, s, 31-H), 1.34 (3H, s, 32-H). ^{13}C NMR δ_{CDCl_3}: 34.3, 28.1, 78.3, 38.8, 45.3, 27.6, 66.8, 156.8, 142.7, 45.5, 197.8, 50.3, 38.6, 59.4, 207.6, 40.9, 49, 16.9, 17.4, 31.9, 19.6, 49.1, 217.5, 46.5, 34.8, 26.6, 179.5, 15.4, 24.4, 18.4.

Ganoderic acid A ESI-MS m/z: 515 $[M-H]^-$, 1031$[2M-H]^-$, 497, 435, 405, 301, 285. ^{1}H NMR δ_{CDCl_3}: 4.62(1H, t, J=8.0Hz, 7-H), 4.79 (1H, t, J=8.0Hz,15-H), 0.98 (3H, s, 18-H), 1.25 (3H, s, 19-H), 0.88 (3H, d, J=6.0Hz, 21-H), 1.24 (3H, d, J=7.3Hz, 26-H), 1.1 (3H, s, 30-H), 1.12 (3H, s, 31-H), 1.28 (3H, s, 32-H). ^{13}C NMR δ_{CDCl_3}: 35.6, 34.3, 208.5, 46.8, 48.9, 29.2, 68.9, 159.0, 140.6, 46.6, 200.2, 51.7, 38.0, 54.0, 72.5, 36.3, 47.9, 17.3, 19.5, 32.6, 19.3, 49.5, 216.9, 46.4, 34.5, 27.3, 179.8, 16.9, 20.7, 19.7.

Ganoderic acid H ESI-MS m/z: 571 $[M-H]^-$, 1143$[2M-H]^-$, 553, 511,467, 437, 319, 303, 301. ^{1}H NMR δ_{CDCl_3}: 3.25 (1H, dd, J=7.6, 8.8Hz, 3-H), 5.63 (1H, s, 12-H), 0.82 (3H, s, 18-H), 1.33 (3H, s, 19-H), 0.99 (3H, d, J=6.4Hz, 21-H), 1.21 (3H, d, J=8.0Hz, 27-H), 1.03 (3H, s, 30-H), 0.88 (3H, s, 31-H), 1.73 (3H, s, 32-H). ^{13}C NMR δ_{CDCl_3}: 33.2, 27.3, 77.2, 40.4, 51.4, 36.6, 198.8, 151.7, 145.7, 39.1, 193.9, 79.2, 47.9, 58.4, 205.7, 37.9, 44.7, 12.2, 17.9, 29.4, 21.6, 48.3, 207.4, 46.4, 34.3, 176, 16.9, 27.9, 15.5, 21.3

Ganoderenic acid D ESI-MS m/z: 511 $[M-H]^-$, 1023$[2M-H]^-$, 493, 449, 301, 285, 247, 149. ^{1}H NMR δ_{CDCl_3}: 4.87 (1H, dd, J=7.6, 9.2Hz,7-H), 0.89 (3H, s, 18-H), 1.24 (3H, s, 19-H), 2.18 (3H, s, 21-H), 6.05 (1H, s, 22-H), 1.23 (3H, d, J=5.6Hz, 27-H), 1.13 (3H, s, 30-H), 1.11 (3H, s, 31-H), 1.40 (3H, s, 32-H). ^{13}C NMR δ_{CDCl_3}: 35.6, 34.3, 216.5, 46.7,

48.8, 27.6, 66.3, 157.4, 141.4, 38.2, 197.8, 48.9, 45.9, 58.6, 216.4, 37.8, 49.7, 19.0, 18.1, 153.8, 21.0, 124.6, 196.1, 47.5, 34.6, 180.6, 16.9, 27.0, 20.8, 24.7.

Ganoderic acid D ESI-MS m/z: 513 $[M-H]^-$, 1027$[2M-H]^-$, 495, 451, 301, 285, 247, 149. ^{1}H NMR δ_{CDCl_3}: 4.80 (1H, t, J=8.8Hz, 7-H), 0.99 (3H, s, 18-H), 1.20 (3H, s, 19-H), 0.94 (3H, d, J=7.6Hz, 21-H), 1.21(3H, d, J=7.6Hz, 27-H), 1.06 (3H, s, 30-H), 1.03 (3H, s, 31-H), 1.30 (3H, s, 32-H). ^{13}C NMR δ_{CDCl_3}: 35.6, 34.3, 217.6, 46.8, 48.9, 27.6, 66.3, 157.8, 141.2, 38.2, 197.6, 50.1, 45.0, 59.3, 216.7, 41.0, 45.6, 17.6, 18.1, 32.0, 19.6, 48.9, 207.6, 46.6, 34.3, 179.4, 16.9, 27.0, 20.8, 24.7.

5.3.3 Cytotoxicity of *Ganoderma lucidum* Triterpenoids on Caco-2, Hep G2 and Hela cells

The effects of 7 triterpenoids isolated from *Ganoderma lucidum* on Caco-2, Hep G2 and HeLa cell growth are shown in **Figure 12**. The LC50 of each triterpenoids in each cells line was calculated as previously described in chapter 3 [131] and summarized in **Table 10**. As shown in **Figure 12** and **Table 10**, ganoderic acid A, G and H did not effectively inhibit cell growth in the three cell lines up to a concentration of 0.70 mg/mL. Ganoderenic acid D was the most cytotoxic and had the lowest LC50s of 0.14±0.011, 0.18±0.022 and 0.26±0.025 mg/mL in Hep G2 cells, Hela cells and Caco-2 cells respectively followed by ganoderic acid I and ganoderic acid D which had similar LC50s in the three cell lines tested. Ganoderic acid B showed selective cytotoxicity to Caco-2 cells (0.36±0.070 mg/mL) and HepG2 cells (0.65±0.051 mg/mL) but not HeLa cells. The variation of LC50s of the 7 compounds in the three cell lines indicated that effects of these triterpenoids are cell specific. The respective chemical structures of the 7 compounds are shown in **Figure 10 (c).** Compounds such as ganoderenic acid D that have an additional double bond between C-20 and C-22 in a triterpenoid structure are likely

relate to the increase cytotoxicity. This finding is consistent with the reported structure-activity relationship that the unsaturation of the side chain of *Ganoderma lucidum* alcohol derivatives is essential for the inhibitory effects of cell proliferation on LNCaP human prostate carcinoma cells [132].

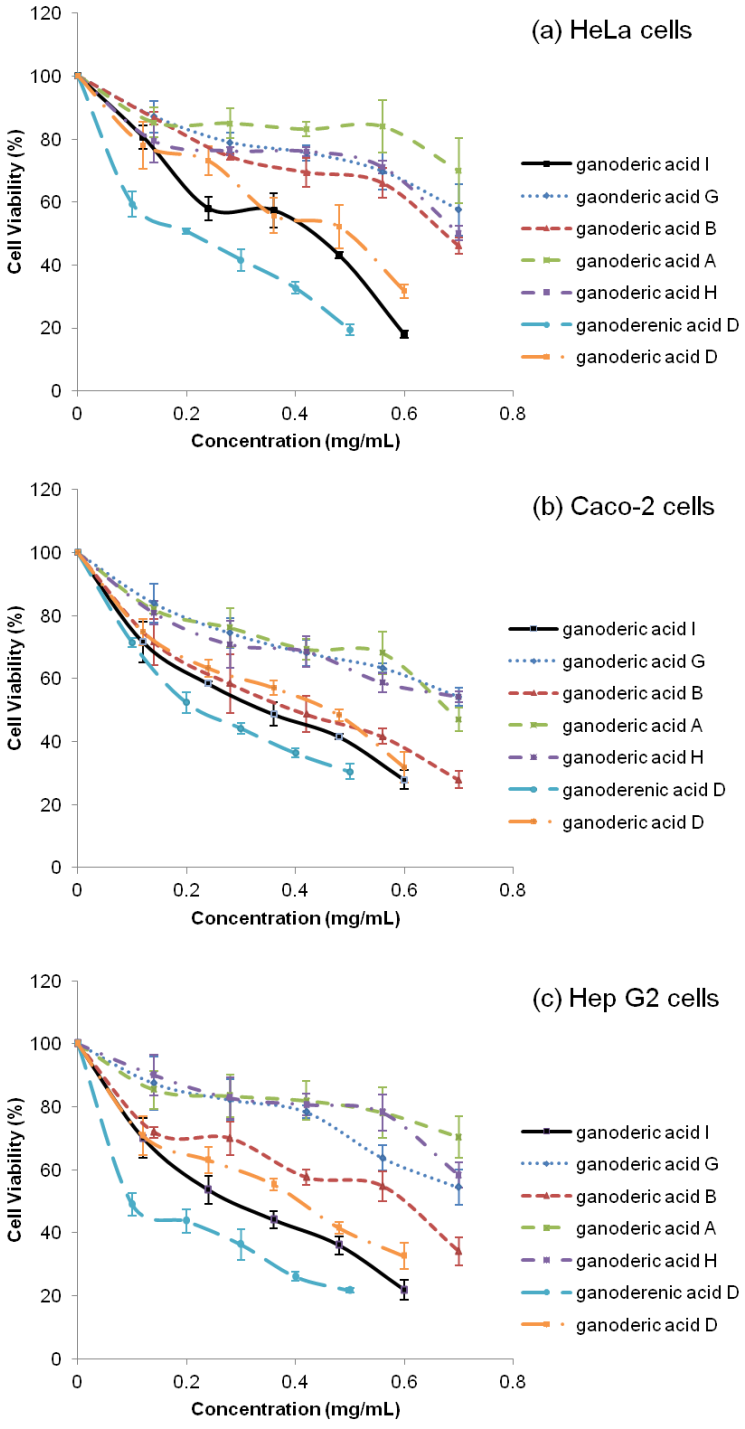


Figure 12. Does-response relationship of ganoderic acid A, B, D, G, H and I and ganoderenic acid D in **(a)** HeLa cells, **(b)** Caco-2 cells and **(c)** Hep G2 cells after 72 h treatment measured by MTT assay. Values are expressed as mean ± SD.

Table 10. Cytotoxicities of 7 triterpenoids isolated from *Ganoderma lucidum* on three human carcinoma cell lines obtained by MTT assay for 72 h treatment. Values are expressed as mean ± SD.

Compound	LC50 of Cell lines (mg/mL)		
	Hep G2	HeLa	Caco-2
ganoceric acid I	0.26±0.037	0.33±0.018	0.39±0.018
ganoderic acid B	0.65±0.051	> 0.70	0.36±0.070
ganoderic acid G	> 0.70	> 0.70	> 0.70
ganoderic acid A	> 0.70	> 0.70	> 0.70
ganoderic acid H	> 0.70	> 0.70	> 0.70
ganoderenic acid D	0.14±0.011	0.18±0.022	0.26±0.025
ganoderic acid D	0.38±0.022	0.37±0.049	0.34±0.005

The LC50 of ganoderenic acid D in Caco-2 cell was half the LC50 (0.528 ± 0.078 mg/mL) we reported for a fractioned *Ganoderma lucidum* triterpenoid extract (fraction 2) in chapter 3 [133]. Ganoderic acid B, I and D were also more toxic than the reported *Ganoderma lucidum* triterpenoid extract in Caco-2 cells. Pure triterpenoids seem to possess more cytotoxicities than a triterpenoid mixture.

A range of LC50s for these triterpenoids has been reported in the literature and are likely cell dependent. For example, in HeLa cells the LC50s of these 7 triterpenoids were reported to range from 8.72 to 20.3 μM which was one of twentieth the LC50s reported in this study. Alternatively an alcoholic extract from *Ganoderma lucidum* spores were shown to have an LC50 of 4.70 mg/mL, which is 10 times higher than the LC50s reported herein [39;119;134;135]. We used pure ganoderic acid H with a purity of 98% as a positive control in HeLa cells to benchmark the fractions cellular response. The dose-response curve and LC50 of the standard were consistent with the dose-response curve of ganoderic acid H that was isolated in this study.

5.4 Conclusions

The RSM was applied to optimize extraction conditions of triterpenoids from *Ganoderma lucidum* for the first time with sufficient simulation modeling and predictions. The optimal extraction conditions for extracting total extract and ganoderic acid H were 98.59% ethanol at 65.15 °C for 5.99 h and 100.00% ethanol at 60.22 °C for 6.00 h respective. We used the optimized extraction conditions of ganoderic acid H to obtain a ganoderic acid enriched crude extract and the crude extract under this condition was further separated by flash chromatography and semi-preparative HPLC to obtain 7 individual triterpenoids. Three of the triterpenoids including ganoderic acid D and I and ganoderenic acid D were found to be cytotoxic does-dependently in Hep G2, HeLa and Caco-2 cells. An additional triterpenoid (ganoderic acid B) was also able to inhibit cell growth in Caco-2 cells and Hep-G2 but not in HeLa cells, indicating a cell-specific inhibitory effect. The most cytotoxic compound was ganoderenic acid D and influence cell growth in all cell lines and is likely a result of an addition double bond in its side chain structure compared to the other compounds tested.

CHAPTER 6 CYTOTOXIC TRITERPENOIDS AND ALCOHOL DERIVATIVES FROM GANODERMA LUCIDUM SHOW VARIOUS EFFECTS ON CELL CYCLE DISTRIBUTION AND APOPTOSIS IN THREE CULTURED HUMAN CARCINOMA CELLS

Preface

Selected portion of Chapter 6 are ready to be submitted as:

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6.1 Introduction

Triterpenoids and their alcohol derivatives from *Ganoderma lucidum* have garnered a lot of attention, as they are believed to contribute to the multiple pharmaceutical properties of this medicinal mushroom. These properties include anti-inflammatory, anti-tumor, anti-androgenic, anti- aldose-reductase and many other biological activities [7;132;136]. So far, hundreds of triterpenoids and their derivatives have been discovered and isolated from *Ganoderma lucidum* [65]. They are lanostane-type triterpenoids with attachment of various functional groups at different positions. Extracts of these triterpenoids and their derivatives have been reported to possess potent chemo-preventative potential affecting multiple regulatory mechanisms in cultured human carcinoma cell models [7]. These involved induction of autophagic and apoptotic cell deaths in human colon carcinoma cells, multidrug-resistant small-cell lung carcinoma cells, human gastric carcinoma cells and inhibition of metastasis in human hepatoma cells [47;48;137-139]. However, the specific bioactivity resulting from individual triterpenoids has not been effectively established. Research is slowed due to the complex and diverse chemical structures and wide variation in the active compounds. Furthermore, there are

many unidentified molecules presented in various extracts that can also contribute to the bioactivity. Elucidating the structure-activity relationship of these triterpenoids is vital for a comprehensive understanding of their bioactivities and mechanism of action. Slight modifications of chemical structure can influence bioactive response. For example, a carboxyl group on the side chain of *Ganoderma lucidum* triterpenoids has been suggested to be the essential functional group for exhibiting aldose reductase inhibitory activity; Ganoderic acid Df was found to inhibit aldose reductase to a greater extent compared to its methyl ester derivatives *in vitro* in which the carboxyl groups have been replaced [136]. Similarly, the number of hydroxyl groups at the side chain and C-3 carbonyl group of *Ganoderma lucidum* alcohol derivatives have been found to be crucial for their anti-androgenic effects and the unsaturation of the side chain to be necessary for suppression of cell growth in LNCaP human prostate carcinoma cells [132]. In chapter 3, we reported that *Ganoderma lucidum* triterpenoids reduced cultured Caco-2 cell growth and these effects were inversely correlated to the compound polarity and the C-23 functional groups on the side chain in Caco-2 human colon carcinoma cells [140]. Therefore, in this chapter, the cytotoxic triterpenoids and the alcohol derivatives from *Ganoderma lucidum* were isolated using flash chromatography and semi-preparative HPLC and their associated bioactivities were investigated in three cultured human carcinoma cell models to evaluate possible structure-activity relationship.

6.2 Materials and Methods

6.2.1 Extraction and Isolation of Triterpenoids and Alcohol Derivatives

The dry *Ganoderma lucidum* was purchased locally (slice of *Ganoderma lucidum*, *chizhi*, origin from China) and ground into a powder and the powder was refluxed with ethanol and separated by flash chromatography isolation as previously described in chapter 3 [141]. The triterpenoid-enriched fraction that had greatest cytotoxicity (fraction

3), was further separated by semi-preparative HPLC with a Waters system (Milford, MA, USA) coupled with Waters 515 HPLC pump, Waters 717 plus autosampler and Waters 2996 PDA detector. A Waters XTerra Prep RP-18 column (7.8 mm × 100 mm, 5 μm particle size) (Milford, MA, USA) was applied with a flow rate of 2 ml/min. Gradient separation was employed with a combination of de-ionized water (A) and acetonitrile (B) as followed: 35% B - 45% B at 0 -5 min, 45 - 55% B at 5 - 15 min, 55% B - 70% B at 15 -35 min, 70% B - 100% B at 35 -40 min and 100% B at 40 - 55 min. Samples were dissolved in 50% ethanol with a concentration of 10 mg/mL and an injection volume of 200 μL was used. Individual triterpenoids and alcohol derivatives were collected according to the UV absorbance and each fraction was evaporate and lyophilized. For bioactivity tests, samples were dissolved in culture media containing 0.4% DMSO and passed through a 0.2 μm filter (Millex GP).

6.2.2 Identification of Chemical Structures by ESI-MS and NMR

To measure the molecular weights of triterpenoids and alcohol derivatives from *Ganoderma lucidum,* ESI-MS detection was conducted using a Bruker amaZonX IonTrap Mass Spectrometer (Bruker Daltonics Inc.). The ESI-MS conditions were set as follows: dry gas flow rate was at 8.0 mL/min; capillary voltage was at 4.5 kV; end plate offset was at -500 V, capillary temperature was set at 250 ^{o}C; flow rate was 0.2 mL/min and injection volume was 5 μL. Both positive and negative ionization was used and scanning mass spectra was ranged from 70 to 2000 m/z.

To identify the chemical structures of triterpenoids and the alcohol derivatives, 2-3 mg samples was dissolved in 600 μL deuterated chloroform and went through NMR analysis with a Bruker AMX500 MHz NMR Spectrometer (Bruker BioSpin GmbH). ^{1}H and DEPT ^{13}C NMR spectra were measured and chemical shifts were expressed in terms of δ (ppm).

6.2.3 Cell Culture

The three human carcinoma cells (Hep G2 cells, Caco-2 cells and HeLa cells) were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured using DMEM (Caisson Laboratories, Inc.) with supplement of 10% fetal bovine serum (Hyclone UK Ltd.) and 100 units/mL of penicillin /streptomycin (Gibco, Invitrogen). The cells were maintained at a concentration between 2×10^5 and 1×10^6 cells/mL and sub-cultured every 3 - 4 days after confluency with 0.25% (w/v) trypsin - 0.53 mM ethylenediaminetetraacetic acid solution (Gibco, Invitrogen). Viable cells were assessed by staining cells with 0.04% trypan blue exclusion dye (MP Biomedicals) and counted in a hemocytometer.

6.2.4 MTT Assay and Cell Cycle Analysis

Effects of *Ganoderma lucidum* triterpenoids and the alcohol derivatives on cell growth were analyzed using MTT assay as previously described in chapter 5. Briefly, a seeding concentration of 2.0×10^4, 2.5×10^4 and 1.5×10^4 cells/mL for Caco-2, Hep G2 and HeLa cells respectively were used in 96-well plates. Cells were treated with each compound at various concentrations for 72 h after 24 h adherence. Untreated cells acted as controls. The media was removed and replaced by MTT (Sigma) after treatments and the resulting formazan crystals were dissolved with SDS (10%) in HCl (0.01 N). The optical density was measured at absorbance of 570 nm and 650 nm by a micro-plate reader (Multiskan Spectrum).

For cell cycle analysis, cells were seeded in 6-well plates at concentrations of 2.0×10^4, 2.5×10^4 and 1.5×10^4 cells/mL for Caco-2, Hep G2 and HeLa cells respectively and treated with each compound for 72 h after 24 h adherence. Untreated cells acted as controls. After treatments, cells were harvested, fixed and stained as previous described in

chapter 3 [142]. Samples were analyzed by Guava PCA flow cytometry with CytoSoft software (Guava Technologies, Inc.).

6.2.5 TUNEL Assay

To quantify the DNA fragmentation induced by apoptotic cell death, TUNEL assay was conducted using a commercial detection kit as previous described in chapter 3 [143].

6.2.6 Statistical Analysis

Results of MTT assay, cell cycle analysis and TUNEL assay were expressed as means ± SD. ESI-MS detection, MTT assay, cell cycle analysis and TUNEL assay were conducted in three separate experiments with three replicate. One-way ANOVA with Duncan post hoc comparison of means and independent-samples T test were used for statistical analysis. Significance was determined at $p < 0.05$ using the SPSS statistical software (v12.0, Chicago, IL, USA).

6.3 Results

6.3.1 Isolation and Identification of Triterpenoids and the Alcohol Derivatives

The representative HPLC chromatograph for isolation of triterpenoids and the alcohol derivatives is shown in **Figure 13**. Each peak was collected as one fraction and a total of 6 fractions were identified as single compounds by ESI-MS and NMR analysis. Chemical structures of these compounds were identified by comparing their molecular weights and chemical shifts to the report literature values [5;74;76;144;145]. The chemical structures of identified compounds are shown in **Figure 14** and the ESI-MS and NMR data are as follow (NMR spectrums are included in Apendix 5):

ganolucidic acid E ESI-MS m/z: 485.6 $[M+H]^+$, 483.3 $[M-H]^-$, 967.6$[2M-H]^-$. ^1H NMR δ_{CDCl_3}: 2.74 (1H, d, J=17.5Hz, 12-H), 4.39 (1H, dd, J=5.7, 9.5Hz, 15-H), 0.89 (3H,

s, 18-H), 1.12 (3H, s, 19-H),0.87 (3H, d, J=5.7Hz, 21-H), 1.12 (3H, s, 28-H), 1.08 (3H, s, 29-H), 1.18 (3H, s, 30-H). DEPT ^{13}C NMR δ_{CDCl_3}: 35.1 (1-C), 34.2 (2-C), 51.5 (5-C), 18.7 (6-C), 29.6 (7-C), 51.8 (12-C), 73.0 (15-C), 38.8 (16-C), 48.9 (17-C), 17.2 (18-C), 18.8 (19-C), 35.9 (20-C), 18.0 (21-C), 34.5 (22-C), 25.7 (23-C), 144.7 (24-C), 12.2 (27-C), 27.8 (28-C), 20.6 (29-C) , 19.0 (30-C).

lucidumol A ESI-MS m/z: 473.7 $[M+H]^+$, 471.3 $[M-H]^-$. DEPT ^{13}C NMR δ_{CDCl_3}: 35.4 (1-C), 34.4 (2-C), 50.4 (5-C), 37.2 (6-C), 23.9 (11-C), 30.1 (12-C), 28.7 (15-C), 31.9 (16-C), 49.0 (17-C), 15.9 (18-C), 24.9 (19-C), 36.6 (20-C), 18.9 (21-C), 33.5 (22-C), 28.6 (23-C), 79.6 (24-C), 23.2 (26-C), 26.6 (27-C), 25.3 (28-C), 21.4 (29-C) , 17.9 (30-C).

ganodermanontriol ESI-MS m/z: 531.8 $[M-H]^-$. DEPT ^{13}C NMR δ_{CDCl_3}: 36.6 (1-C), 34.9 (2-C), 50.7 (5-C), 23.7 (6-C), 120.0 (7-C), 117.2 (11-C), 37.8 (12-C), 27.9 (15-C), 31.5 (16-C), 51.0 (17-C), 15.7 (18-C), 22.5 (19-C), 36.5 (20-C), 18.6 (21-C), 33.5 (22-C), 28.9 (23-C), 79.3 (24-C), 67.7 (26-C), 22.5 (27-C), 25.5 (28-C), 22.1 (29-C) , 25.4 (30-C).

7-oxo-ganoderic acid Z ESI-MS m/z: 471.7 $[M+H]^+$, 470.0 $[M-H]^-$, 939.7 $[2M-H]^-$. DEPT ^{13}C NMR δ_{CDCl_3}: 34.8 (1-C), 27.4 (2-C), 49.8 (5-C), 36.6 (6-C), 23.7 (11-C), 30.1 (12-C), 32.0 (15-C), 28.8 (16-C), 48.9 (17-C), 15.8 (18-C), 18.4 (19-C), 36.2 (20-C), 18.6 (21-C), 34.8 (22-C), 25.9 (23-C), 145.5 (24-C), 12.1 (27-C), 25.0 (28-C), 27.4 (29-C) , 15.3 (30-C).

15-hydroxy-ganoderic acid S ESI-MS m/z: 467.8 $[M-H]^-$, 935.6 $[2M-H]^-$. DEPT ^{13}C NMR δ_{CDCl_3}: 36.6 (1-C), 34.8 (2-C), 50.5 (5-C), 23.6 (6-C), 121.2 (7-C), 116.9 (11-C), 38.5 (12-C), 74.6 (15-C), 40.0 (16-C), 48.9 (17-C), 16.0 (18-C), 22.2 (19-C), 35.9 (20-C), 18.3 (21-C), 34.7 (22-C), 25.8 (23-C), 145.1 (24-C), 12.1 (27-C), 17.0 (28-C), 25.4 (29-C) , 22.5 (30-C).

ganoderic acid DM ESI-MS m/z: 469.7 $[M+H]^+$, 937.4$[2M+H]^+$,467.3 $[M-H]^-$, 935.7$[2M-H]^-$. DEPT ^{13}C NMR δ_{CDCl_3}: 35.4 (1-C), 34.4 (2-C), 50.4 (5-C), 37.2 (6-C), 23.8 (11-C), 30.1 (12-C), 28.7 (15-C), 31.9 (16-C), 49.0 (17-C), 15.9 (18-C), 17.9 (19-C), 36.2 (20-C), 18.6 (21-C), 34.7 (22-C), 25.9 (23-C), 145.4 (24-C), 12.1 (27-C), 25.4 (28-C), 22.4 (29-C) , 24.9 (30-C).

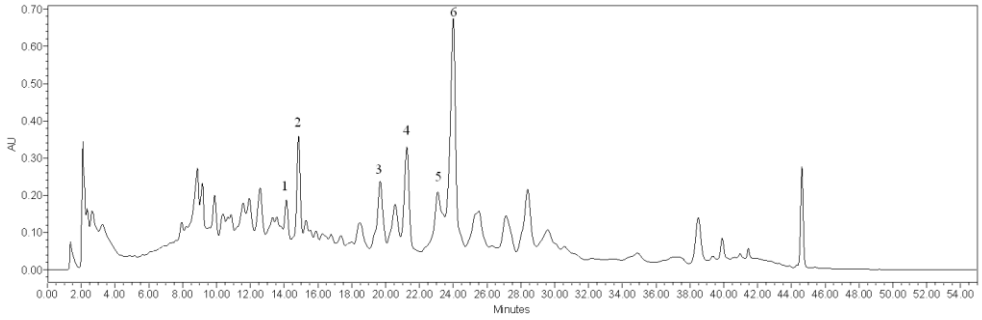
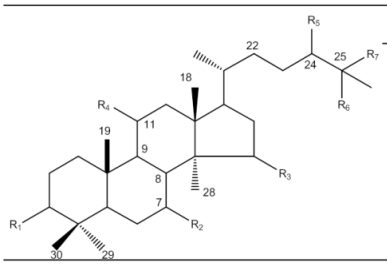


Figure 13. Representative HPLC chromatograph of fraction 3 from *Ganoderma lucidum* by semi-preparative HPLC. Peak numbers refer to the identified individual triterpenoids and the alcohol derivatives. 1, ganolucidic acid E; 2, lucidumol A; 3, ganodermanontriol; 4, 7-oxo-ganoderic acid Z; 5, 15-hydroxy-ganoderic acid S; 6, ganoderenic acid DM.



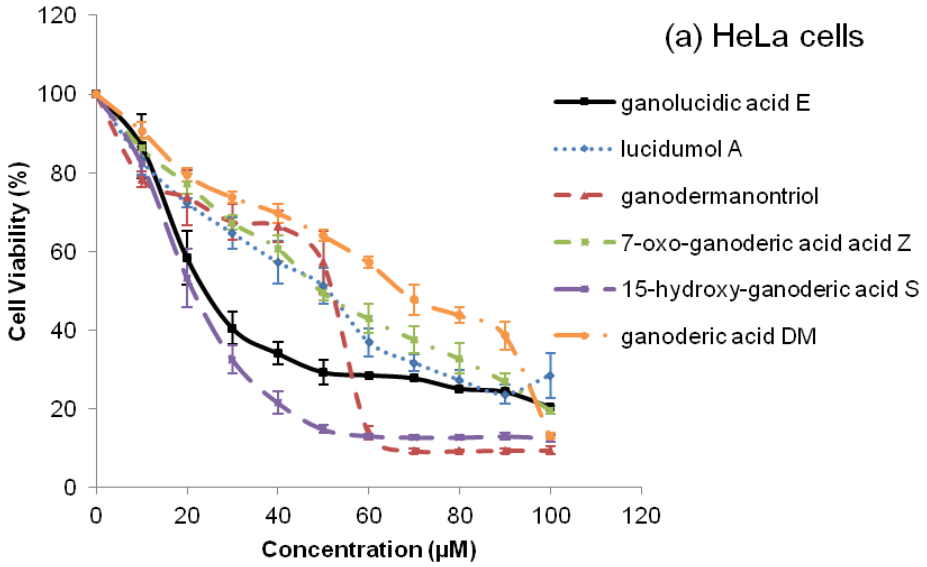
compound	R1	R2	R3	R4	R5	R6	R7	double bond
ganolucidic acid E	O	H	OH	O	H	COOH	H	8,9;
lucidumol A	O	O	H	H	OH	OH	CH3	8,9;
ganodermanotriol	O	H	H	H	OH	OH	CH2OH	7,8;9,11
7-oxo-ganoderic acid Z	OH	O	H	H	H	COOH	H	8.9;24,25
15-hydroxy-ganoderic acid S	O	H	OH	H	H	COOH	H	8,9;24,25
ganoderic acid DM	O	O	H	H	H	COOH	H	8,9;24,25

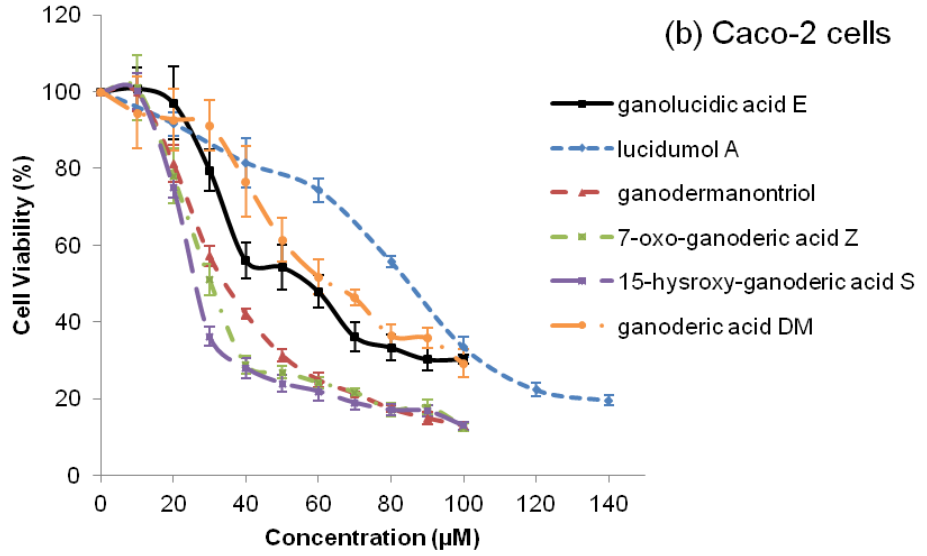
Figure 14. Chemical structures of identified compounds from *Ganoderma lucidum*.

6.3.2 The Effects of *Ganoderma lucidum* Triterpenoids and the Alcohol Derivatives on Three Human Carcinoma Cell Growth

The effect of isolated triterpenoids and the alcohol derivatives from *Ganoderma lucidum* on Caco-2, Hep G2 and HeLa cell growth are shown in the dose-response curves in **Figure 15**. The LC50 values are determined by plotting cell viabilities against log

concentrations (graph not shown) and shown in **Table 11**. All six compounds are able to inhibit cell growth in a dose dependent manner in all three cell lines. Hep G2 cells are the most sensitive cell line with less cell variability at the same concentration treatment compared to the other two cell lines. Ganodermanontriol had the greatest cell growth inhibitory effect on Hep G2 cell with an LC50 of 20.87 ± 1.48 µM, while 15-hydroxyl-ganoderic acid S exhibits the most cell growth inhibitory effect in HeLa and Caco-2 cells with LC50s of 21.17 ± 1.81 and 30.38 ± 1.44 µM respectively.


(a) HeLa cells
Cell Viability (%)
Concentration (μM)
ganolucidic acid E
lucidumol A
ganodermanontriol
7-oxo-ganoderic acid acid Z
15-hydroxy-ganoderic acid S
ganoderic acid DM


(b) Caco-2 cells
Cell Viability (%)
Concentration (μM)
ganolucidic acid E
lucidumol A
ganodermanontriol
7-oxo-ganoderic acid Z
15-hysroxy-ganoderic acid S
ganoderic acid DM

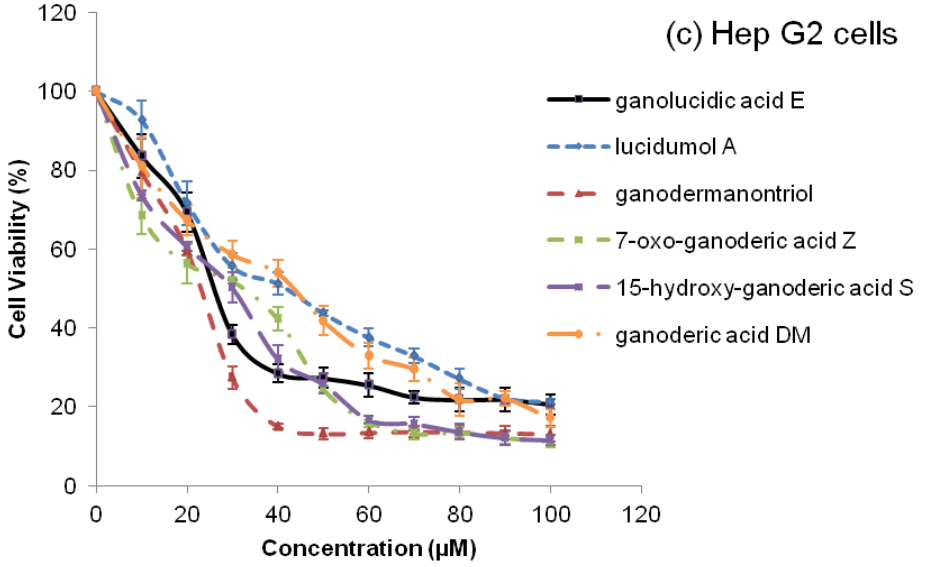

(c) Hep G2 cells
Cell Viability (%)
Concentration (μM)
ganolucidic acid E
lucidumol A
ganodermanontriol
7-oxo-ganoderic acid Z
15-hydroxy-ganoderic acid S
ganoderic acid DM

Figure 15. Dose-response relationship of 6 triterpenoids and the alcohol derivatives from *Ganoderma lucidum* in **(a)** HeLa cells, **(b)** Caco-2 cells and **(c)** Hep G2 cells measured by MTT assay for 72 h treatment. Values are expressed as mean ± SD.

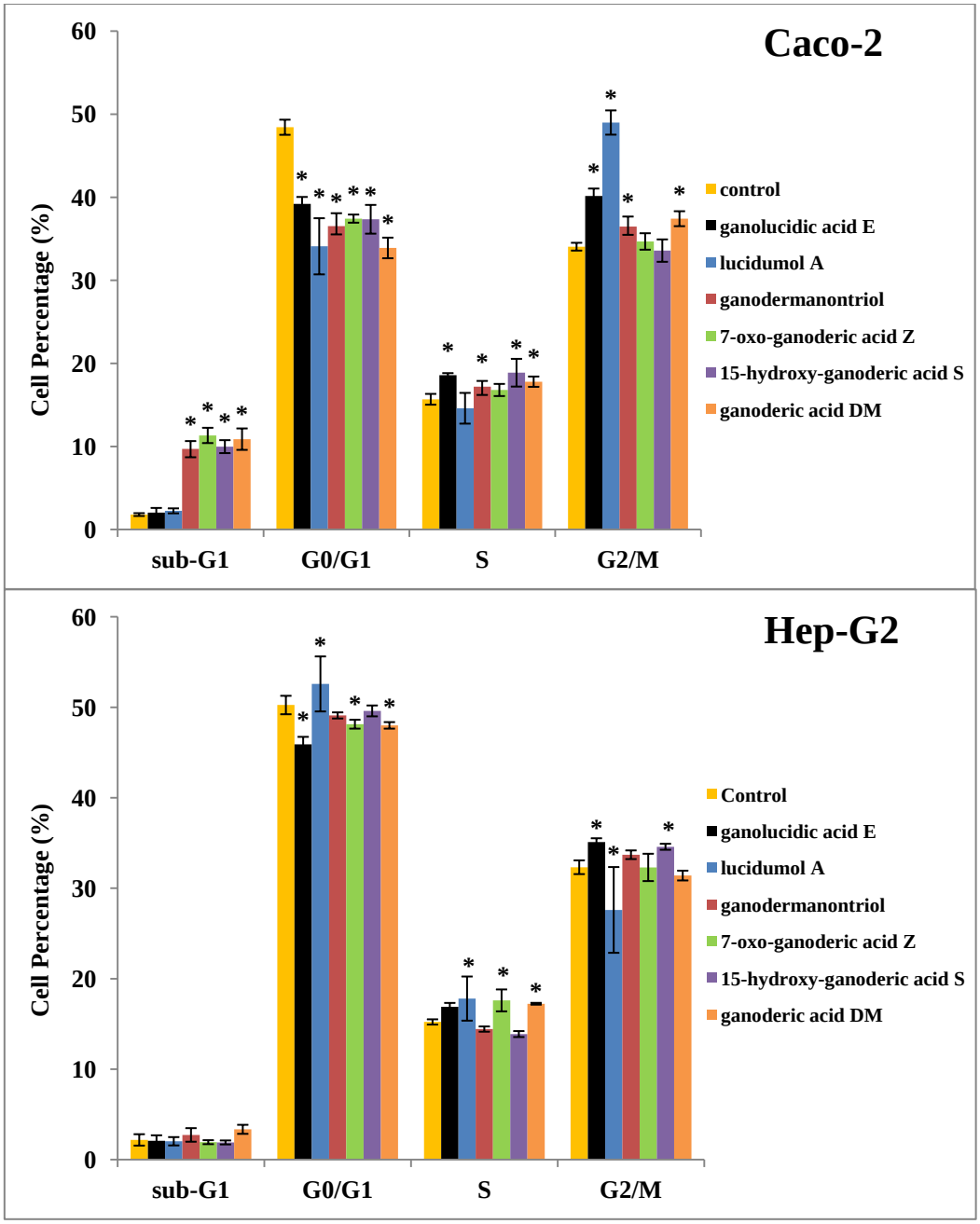
Table 11. Cytotoxicities of 6 triterpenoids and the alcohol derivatives from *Ganoderma lucidum* on three human carcinoma cell lines from MTT assay for 72 h treatment. Values are expressed as mean ± SD.

Compound	LC50s of Cell lines (μM)		
	Heg G2	HeLa	Caco-2
ganolucidic acid E	28.28±2.57	32.25±5.55	53.07±4.07
lucidumol A	38.58±5.27	42.07±4.27	84.36±9.97
ganodermanontriol	20.87±1.48	36.50±4.67	36.77±2.79
7-oxo-ganoderic acid Z	22.08±2.23	45.88±3.29	30.83±2.54
15-hydroxy-ganoderic acid S	21.52±1.81	21.17±1.81	30.38±1.44
ganoderic acid DM	35.34±4.46	55.61±0.88	63.72±8.17

6.3.3 Cell Cycle Distribution Regulated by *Ganoderma lucidum* Triterpenoids and the Alcohol Derivatives

The corresponding cell cycle distributions of three human carcinoma cells treated with the six compounds are shown in **Figure 16**. All six compounds are able to significantly ($p < 0.05$) accumulate cell populations at sub-G1 phase in HeLa cells with an increase percentage range from 5.11% for ganoderic acid DM to a high 22.06% for 15-hydroxy-ganoderic acid S. Four compounds which are ganodermanontriol, 7-oxo-ganoderic acid Z, 15-hydroxy-ganoderic acid S and ganoderic acid DM show significantly ($p < 0.05$) increased sub-G1 cell percentages with a range of 7.90% (ganodermanontriol) to 9.53% (7-oxo-ganoderic acid Z) in Caco-2 cells, while none of the compound caused any significant changes in sub-G1 cell accumulation in Hep G2 cells. All six compounds cause a significant ($p < 0.05$) reduction of cells at G0/G1 phase in both Caco-2 and HeLa cells with decreased percentages ranged from 3.9% (lucidumol A) to 20.6 % (15-hydroxy-ganoderic acid S) but not for treatments with lucidumol A, ganodermanontriol and 15-hydroxy-ganoderic acid S in Hep G2 cells. Conversely,

lucidumol A causes a G0/G1 arrest in Hep G2 cells with an increase in cell percentage of 2.33% compared to the control cells while ganodermanontriol and 15-hydroxy-ganoderic acid S do not cause significant changes at G0/G1 phase in Hep G2 cells. Compared to the control cells, cell percentages in the S phase do not change significantly in HeLa cell, while treatments of ganolucidic acid E, ganodermanontriol, 15-hydroxy-ganoderic acid S and ganoderic acid DM have a significant ($p < 0.05$) increase in cells at S phase in Caco-2 cells. Lucidumol A, 7-oxo-ganoderic acid Z and ganoderic acid DM increase cell percentages at S phase in Hep G2 cells. Treatments of ganolucidic acid E, lucidumol A, ganodermanontriol and ganoderic acid DM cause a G2/M arrest in Caco-2 cells (increment ranged from 2.43% for ganodermanontriol to 14.95% for lucidumol A) and ganodermanontriol and ganoderic acid DM result in G2/M arrest in HeLa cells with increment of 7.10% and 14.20% respectively. Similarly, ganolucidic acid E and 15-hydroxy-ganoderic acid S induce an accumulation of G2/M cells in Hep G2 cells but to a smaller extent compared to similar treatment in the other two cell lines, with increment of 2.78% and 2.28% respectively. Additionally, a significant ($p < 0.05$) reduction in cells at G2/M phases are observed with treatments of ganolucidic acid E and lucidumol A in HeLa cells and treatment of lucidumol A in Hep G2 cells with reduced cell percentages of 2.80% , 3.25% and 4.72% respectively.


Caco-2
Cell Percentage (%)
60
50
40
30
20
10
0
sub-G1
G0/G1
S
G2/M
control
ganolucidic acid E
lucidumol A
ganodermanontriol
7-oxo-ganoderic acid Z
15-hydroxy-ganoderic acid S
ganoderic acid DM
Hep-G2
Cell Percentage (%)
60
50
40
30
20
10
0
sub-G1
G0/G1
S
G2/M
Control
ganolucidic acid E
lucidumol A
ganodermanontriol
7-oxo-ganoderic acid Z
15-hydroxy-ganoderic acid S
ganoderic acid DM

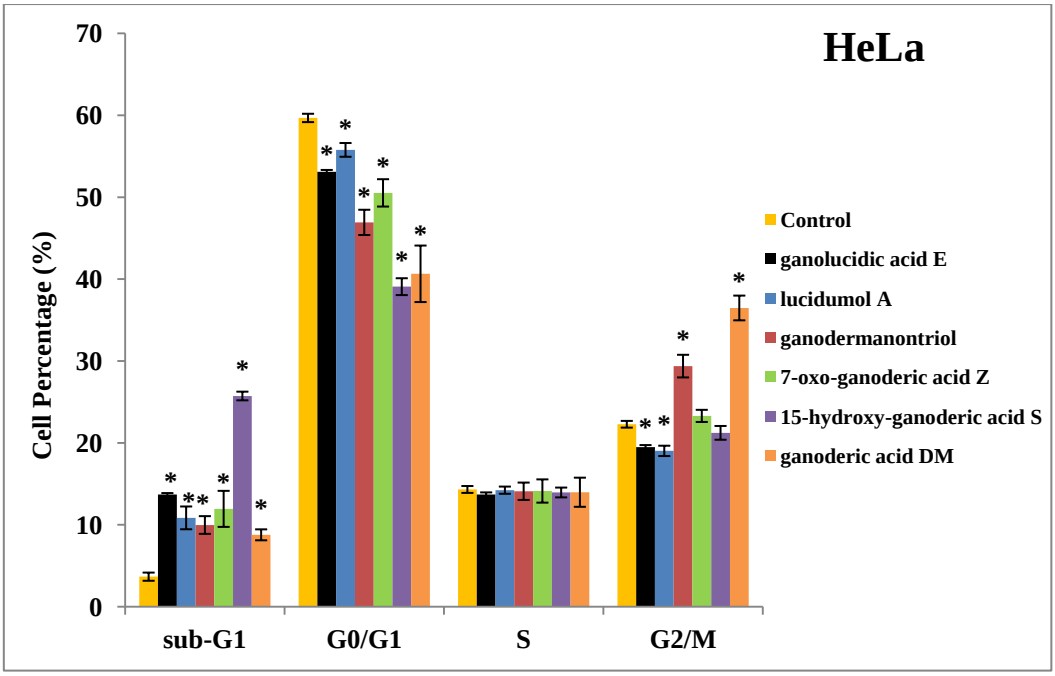
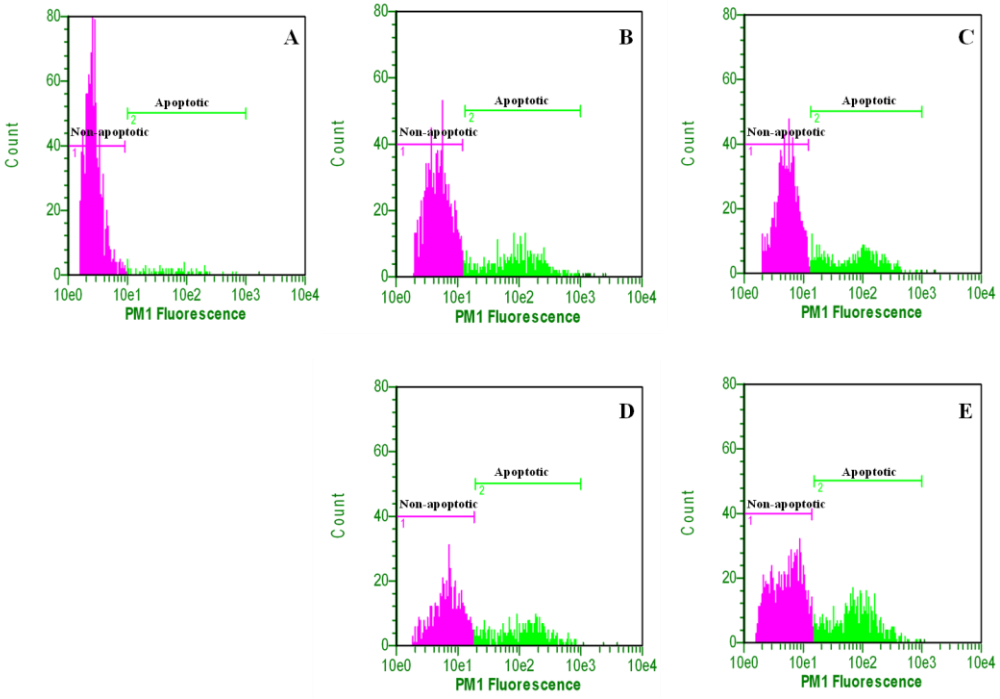


Figure 16. Cell cycle distributions of Caco-2, Hep G2 and HeLa cells for 72 h treatments of 6 triterpenoids and the alcohol derivatives from *Ganoderma lucidum* at their respective LC50 concentrations obtained by MTT assay. Values are expressed as mean ± SD. An asterisk represents significant differences ($p < 0.05$) in the same phases within the same cell line compared to control values.

6.3.4 Induction of Apoptosis by *Ganoderma lucidum* Triterpenoids and the Alcohol Derivatives on Caco-2 and HeLa Cells

Since sub-G1 accumulations were observed with treatments of triterpenoids and the alcohol derivatives in Caco-2 and HeLa cells, which indicated possible involvement of DNA fragmentation in the process of cell death, the TUNEL assay was conducted to quantify the resulting apoptotic DNA fragments. Results of the flow cytometry analysis are shown in **Figure 17** and the cell percentages of apoptotic and non-apoptotic populations are summarized in **Table 12**. All tested compounds induce a significant ($p < 0.05$) increase apoptotic cell percentage from 3.47% to 43.03% and a corresponding reduction of non-apoptotic cell percentages compared to untreated control cells in both cell lines. 15-hydroxy-ganoderic acid S is the most potent apoptotic inducer while ganodermanontriol exhibits the least potency in HeLa cells.


A
B
C
D
E
Apoptotic
Non-apoptotic
Count
PM1 Fluorescence
10e0
10e1
10e2
10e3
10e4

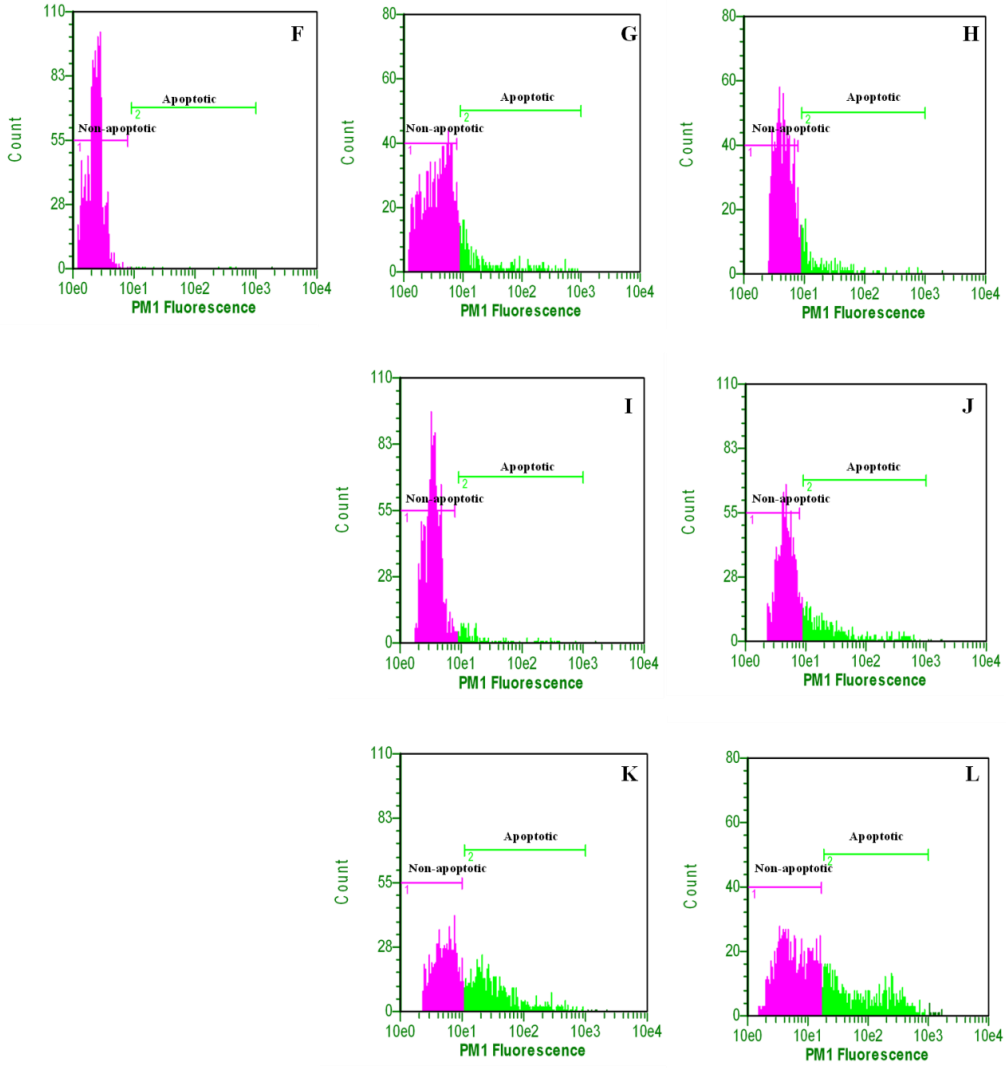


Figure 17. Representative flow cytometry analysis of TUNEL assay for 72 h treatments of identified triterpenoids and the alcohol derivatives from *Ganoderma lucidum* at their respective LC50 concentrations. **A,** control treatment of Caco-2 cells; **B-E,** treatment of ganodermaonontriol, 7-oxo-ganoderic acid Z, 15-hydroxy-ganoderic acid S and ganoderic acid DM on Caco-2 cells respectively; **F,** control treatment of HeLa cells; **G-L,** treatment of ganolucidic acid E, lucidumol A, ganodermaonontriol, 7-oxo-ganoderic acid Z, 15-hydroxy-ganoderic acid S and ganoderic acid DM on HeLa cells respectively.

Table 12. Cell percentages of TUNEL assay in Caco-2 and HeLa cells for 72 h treatments of triterpenoids and the alcohol derivatives from *Ganoderma lucidum* at their respective LC50 concentrations. Values are expressed as mean ± SD. An asterisk represents significant differences ($p < 0.05$) in the same group within the same cell line compared to control values.

Compound	TUNEL Assay in Caco-2 cells cell percentage (%)		TUNEL Assay in HeLa cells cell percentage (%)	
	Non-apoptotic	Apoptotic	Non-apoptotic	Apoptotic
control	94.05±0.71	5.86±0.71	97.09±1.92	2.86±1.88
ganolucidic acid E	-	-	83.20±0.73*	16.80±0.73*
lucidumol A	-	-	87.78±1.17*	12.06±1.22*
ganodermanontriol	74.34±1.62*	25.45±1.34*	93.50±2.08*	6.33±2.02*
7-oxo-ganoderic acid Z	73.65±1.41*	26.13±1.24*	73.29±0.39*	26.51±0.43*
15-hydroxy-ganoderic acid S	66.60±1.00*	33.09±0.58*	53.70±0.44*	45.90±0.45*
ganoderic acid DM	64.20±3.47*	35.70±3.45*	62.40±0.60*	37.10±0.83*

6.4 Discussions

Six triterpenoids and alcohol derivatives have been isolated from the ethanol extract of *Ganoderma lucidum*. This thesis is the first to report their bioactive effects in three distinct cancer cell models. LC50s of the six compounds in Caco-2, Hep G2 and HeLa cells ranged from 20.87 µM to 84.36 µM. The range of the LC50s is consistent to reported cytotoxicity of *Ganoderma lucidum* triterpenoids in other cell models. For instant, the LC50 of ganoderic acid Me was reported to be 36.9 µM in HCT-116 cells [49]. The LC50s of ganoderic acid Mf and S ranged from 19.5 µM to 39.1 µM in HeLa cells while the LC50 of an alcohol derivative, ganoderiol F was found to be 17 µM in Hep G2 cells [50;52]. The LC50s of all the six individual triterpenoids and alcohol derivatives are much lower compared to the LC50 (0.348 ± 0.032 mg/mL) of their parent crude extract as repoterd in chapter 3 [146]. Among the three cell models, Hep G2 cells was most sensitive to the cytotoxic effects of the six compounds while Caco-2 cells was the most resistant one which was evident by relatively high LC50s for all six compounds. The relatively cytotoxic resistance of HeLa and Caco-2 cells may possibly due to their

aneupliod nature and chromosome instability, compared to a relatively near-diploid karyotpe of Hep G2 cells [147]. Aneuploid and chromosome instability were believed to contribute to the acquisition of drug resistance by enhancing adaptation capacity to withstand cellular stresses [148;149]. LC50s of the six compounds in Hep G2 cells were found to be in the following order: ganodermanontriol < 15-hydroxy-ganoderic acid S < 7-oxo-ganoderic acid Z < ganolucidic acid E < ganoderic acid DM < lucidumol A. While in HeLa cells, it was 15-hydroxy-ganoderic acid S < ganolucidic acid E < ganodermanontriol < lucidumol A <7-oxo-ganoderic acid Z < ganoderic acid DM. In addition, in Caco-2 cells, the order was 15-hydroxy-ganoderic acid S < 7-oxo-ganoderic acid Z < ganodermanontriol < ganolucidic acid E < ganoderic acid DM < lucidumol A. The order of relative potency in the six compounds seemed to be similar in Hep G2 and Caco-2 cells while a completely different order was observed in HeLa cells, suggesting that effects of six triterpenoids and alcohol derivatives are likely cell specific. Though no obvious structured-activity relationship was observed, it was noticed that ganoderic acid DM and lucidumol A which were less cytotoxic in all three cell lines both have oxy groups at position C-3 and C-7 of the ring structure. The oxy groups in these two positions are likely to interfere with the biding capacity of triterpenoids to cellular constituents possibly protein domains that could trigger the toxic effects and thus contribute to reduced cytotoxicity. Similar evidence of structure-activity relationship has been reported for 3-oxo ursolic acid derivative of ursolic acid exhibited less cytotoxicity in HeLa cells than ursolic acid. Ursolic acid has a hydroxyl group at C-3 and a hydrogen donor group at either C-3 and/or C-28 and this has been reported to be essential for the cytotoxic properties [150]. Multiflorane-type triterpenoids derived from Trichosanthes kirilowii with an oxy group at C-3 had impaired activity compared to a hydroxyl group. Furthermore, the presence of oxy group at either C-3 and/or C-11 reduced the inhibitory

effects of these triterpenoids on Epstein–Barr virus early antigen activation induced by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate in Raji cells [151].

Similar to the cytotoxic effects of the triterpenoids and alcohol derivatives, their effects on cell cycle distributions were variable and different with respects to structure and cell line. For example, lucidumol A caused an accumulation of sub-G1 cell populations in HeLa cells but not in Caco-2 and Hep G2 cells. Lucidumol A also caused a G0/G1 arrest in Hep G2 cells while another alcohol derivative ganodermanontriol did not affect the cell cycle distribution in the same cell line. Similarly, ganoderic acid Mf was reported to cause a G0/G1 arrest while ganoderic acid S triggered a cell cycle arrest at S phase in HeLa cells [50]. Conversely, none of the six sample treatments in our study caused either G0/G1 or S phase arrest in HeLa cells but instead caused significant reductions in the percentage of cells at the G0/G1 phase. Similar to our reported G0/G1 arrest from lucidumol A in Hep G2 cells, another alcohol derivative, ganoderiol F, was reported to cause a cell cycle arrest at the G0/G1 phase [52]. No conclusive structure-activity relationship could be observed in this study. However, the number of hydroxyl groups on the side chain for ganoderma alcohols seem to influence the cell cycle distribution in Hep G2 cells, in which ganoderma alcohols with two hydroxyl groups on the side chain caused G0/G1 arrest while those with three hydroxyl groups do not. It was noticed that treatments using all six compounds failed to cause any significant accumulated sub-G1 cells in Hep G2 cells even though Hep G2 was the most sensitive cell line. This finding indicated that the cytotoxic effect of *Ganoderma lucidum* triterpenoids and the alcohol derivatives were not necessarily correlated to apoptotic cell death.

In HeLa and Caco-2 cells, results from the TUNEL assay confirmed apoptotic cell death. The percentages of apoptotic cells in all treatments in Caco-2 cells were similar

while percentages of apoptotic cells in treatments in HeLa cells varied. Among the six compounds, ganodermanontriol treated cells increased apoptotic cells by 3.47% compared to control in HeLa cells, indicating the accumulated sub-G1 cells may only be partially attributed to apoptotic cell death.

Six triterpenoids and the alcohol derivatives have been successfully isolated from a *Ganoderma lucidum* extract and their effects on cell growth and apoptosis were measured in three distinct cultured carcinoma cell lines. Theses triterpenoids and alcohol derivatives exhibited diverse regulatory effects in the cell growth and cell cycle distribution in Caco-2, Hep G2 and HeLa cells. Some were able to induce apoptotic cell death in Caco-2 and HeLa cells but not Hep G2 cells. The cell specific effects of these compounds did not show any obvious structure-activity relationship. It is likely, that there is multiple activation of different cellular regulation and the variation is depending on cell type. Further study is needed to elucidate their underlying regulation mechanisms in different cell models. This chapter provides scientific evidence on the bioactivity of six *Ganoderma lucidum* triterpenoids and alcohol derivatives in cultured human carcinoma cell models and contributes to understandings of these compounds as potential chemoprevention agents.

CHAPTER 7 GANODERMA LUCIDUM TRITERPENOIDS AND THE ALCOHOL DERIVATIVES STIMULATE CASPASE ACTIVATION AND CYTOCHROME C RELEASE, INDUCE APOPTOSIS AND ALTER MULTIPLE CELLULAR PATHWAYS IN CULTURED HUMAN CARCINOMA CELLS

Preface

Selected portion of Chapter 7 are ready to be submitted as:

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7.1 Introduction

Apoptosis, also known as programmed cell death, is a mode of cell death without associated inflammation for cell clearance to maintain particular cell number and tissue size in normal tissue development and homoeostasis [152]. The apoptotic cell death is typically characterized by cell shrinkage, nuclear chromatin condensation, DNA fragmentation and formation of apoptotic bodies [153]. To date, two key pathways are believed to be responsible for apoptotic cell death [154-158]. One pathway is the extrinsic pathway, which involves activation of receptors in the death receptors subfamily and subsequent recruitments of initiator caspases. The other pathway, which involves p53 activation, is intrinsic and mediated through mitochondria. Both pathways are able to result in alteration of protein expressions in Bcl-2 family that subsequently cause permeabilization of mitochondrial outer membrane to release multiple apoptogenic proteins including cytochrome c and apoptosis inducing factor and eventually stimulate downstream effector caspases to regulate the process of cell death. Due to the complex and multiple steps involved in the apoptotic pathways, minor alteration in these transduction signaling could impair normal apoptosis regulation and result in

carcinogenesis [159]. Thus, the induction of apoptotic has become the primary strategy for antitumor agents to exert antitumor effect against carcinoma cells in cancer chemotherapy [160-162].

Bioactive components from medicinal plants, particularly phytochemicals, have gained increasing research interests for their effective anticancer properties [163;164]. Growing experimental evidence has indicated that some of the phytochemicals can inhibit cell growth and induce apoptosis in cancer cells *in vitro* [165-168]. Triterpenoids from *Ganoderma lucidum* which is a medicinal mushroom used as folk remedy in ancient Asia, has been reported to possess anti-tumor effects in multiple carcinoma cell lines via diverse regulation mechanisms [7]. A triterpene-enriched extracts from *Ganoderma lucidum* was reported to induce apoptotic cell death in SW620 cells by up regulation of p53, increment of BAX/Bcl-2 ratio and activation of caspase-3 activity [48]. Similar effects were observed in treatments of ganoderic acid Me in HCT-116 cells, ganoderic acid T in 95-D cells and ganoderic acid Mf and S in HeLa cells, all of which stimulated apoptotic cell deaths through a mitochondria-mediated pathway [49;50;169]. However, the possibility of involvement of other regulation pathways and existence of cell-specific effects of these *Ganoderma lucidum* triterpenoids are not well understood. In chapter 6, we have indicated that induction of apoptosis by *Ganoderma lucidum* triterpenoids and the alcohol derivatives was cell specific and likely to involve multiple regulation of cellular signaling. To further elucidate the apoptosis regulation mechanisms by *Ganoderma lucidum* triterpenoids and the alcohol derivatives, this chapter examined the possible involvement of caspase activation, membrane protein externalization and release of cytochrome c and screened for potential cancer-related regulation transcription signaling.

7.2 Materials and Methods

7.2.1 Isolation and Chemical Characterization of *Ganoderma lucidum* Triterpenoids and the Alcohol Derivatives

Six triterpenoids and the alcohol derivatives including ganolucidic acid E, lucidumol A, ganodermanontriol, 7-oxo-ganoderic acid Z, 15-hydroxy-ganoderic acid S and ganoderic acid DM were obtained by flash chromatography and semi-preparative HPLC from an ethanol extract of *Ganoderma lucidum* as previously described in chapter 6. Their molecular weights and chemical structures were determined and confirmed by ESI-MS and ^{1}H and DEPT ^{13}C NMR analysis and compared to references as previously described in chapter 6.

7.2.2 Cell Culture

Three cultured human carcinoma cells (Hep G2 cells, Caco-2 cells and HeLa cells) were originated from American Type Culture Collection (Manassas, VA, USA). They were cultured as stated in chapter 6. Their viabilities were assessed with a staining of 0.04% trypan blue exclusion dye (MP Biomedicals) and counted in a hemocytometer.

7.2.3 Multi-caspase Apoptotic Assay

Multi-caspase apoptotic assay were conducted using a multi-caspase detection kit (Guava Technologies, Inc.) and analyzed on a Guava PCA flow cytometry (Guava Technologies Inc.) as previously described in chapter 3 [170]. Briefly, cells were seeded in 6-well plates and treated with triterpenoids and alcohol derivatives that induce apoptosis at their respective previously reported LC50 concentrations (chapter 6) for 48 h after 24 h adherence. Untreated cells acted as controls. After treatment, cells were harvested by trypsinization and centrifugation. The cell pellets were then washed with PBS, adjusted to a concentration of 5×10^{5} cells/mL and firstly stained with

SR-VAD-FMK reagent (Guava Technologies Inc.) with a subsequent second staining of 7-AAD reagent (Guava Technologies Inc.).

7.2.4 Annexin-V-Fluorescein Isothiocyanate (FITC)/PI Apoptotic Asssay

To assess the translocation of phosphatidylserine during apoptosis, an Annexin V-FITC Apoptosis detection Kit (eBioscience, Inc., San Diego, CA, USA) was used according to the manufacture's instruction with minor modification. Briefly, cells were seeded in 96-well plates and treated with the six triterpenoids and the alcohol derivatives at their respective previously reported LC50 concentrations for 72 h. Untreated cells acted as controls. Cells were washed with the binding buffer and co-stained with 5 µL annexin V-FITC reagent and 10 µg/mL PI at room temperature for 30 min. After staining, cells were visualized by a LSM 510 laser scanning confocal microscope (Carl Zeiss, Heidelberg, Germany) with a a Zeiss LD Plan-Neofluar 20 × objective (Carl Zeiss). The excitation wavelength was set at 488 nm and 543 nm and the emission wavelength was 510 nm and 620 nm for annexin V-FITC and PI respectively.

7.2.5 Detection of Cytochrome-c Release by Confocal Microscope

Cells were grown in 96-well plates and treated with the six triterpenoids and the alcohol derivatives as mentioned above. After treatment, cells were washed with PBS and stained with 300 nM MitoTracker Deep Red FM (Life Technologies, Molecular Probes, Inc., Eugene, OR, USA) in DMEM in a 5% CO_2 incubator at 37°C for 35 min. After staining the mitochondria, cells were washed with PBS and fixed with 4% formaldehyde for 10 min at room temperature, followed by blocking of nonspecific bindings with 5% BSA (Sigma) for 30 min at 37°C in a 5% CO_2 incubator. Cells were then stained with 5 µg Anti-Cytochrome C FITC reagent (eBioscience, Inc.) for each sample for 60 min at 37°C in the incubator. The double-labeled cells were then visualized by a LSM 510 laser

scanning confocal microscope (Carl Zeiss) with a Zeiss LD Plan-Neofluar 20× objective (Carl Zeiss). The excitation wavelength was set at 488 nm and 633 nm and the emission wavelength was 510 nm and 690 nm for Anti-Cytochrome C FITC and MitoTracker Deep Red FM respectively.

7.2.6 Luciferase Reporter Arrays Analysis

7.2.6.1 Transient Transfection

Cells were seeded at concentrations of 5×10^4 and 3×10^5 cells/mL for HeLa and Hep G2 cells respectively in 96-well plates. A Cignal Finder Cancer 10-pathway reporter system (Qiagen, Valencia, CA, USA) which contains an inducible transcription factor-responsive firefly luciferase reporter and the constitutively expressing Renilla construct were employed 24 h after seeding. Transient transfections were conducted using the lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

7.2.6.2 MTT Assay

To determine the doses applied in the luciferase reporter assay, MTT assay was conducted with cell seeding concentrations of 5×10^4 and 3×10^5 cells/mL for HeLa and Hep G2 cells respectively in 96-well plates. Cells were treated with the six triterpenoids and the alcohol derivatives at various concentrations from 20 µM to 400 µM for 18 h after 24 h adherence. Untreated cells acted as controls. After treatment, cells were treated with MTT solution and their viabilities were assessed as previously describe in chapter 3 [171].

7.2.6.3 Luciferase Assay

After transient transfections, cells were treated with six triterpenoids and the alcohol derivatives for 18 h in their respective LC50 concentrations obtained by MTT assay as

mentioned above while untreated cells acted as controls. The cells were then wash with PBS, lysed and the luciferase activities were measured using the Dual Luciferase Assay system (Promega, Madison, WI, USA) with a BioTek Synergy HT Multidetection Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). The luminescent signals of the firefly luciferase were normalized to renilla luciferase by the firefly/renilla ratios and the fold change of relative luciferase units were determined by dividing the firefly/renilla ratios of samples to control cells.

7.2.7 Statistical Analysis

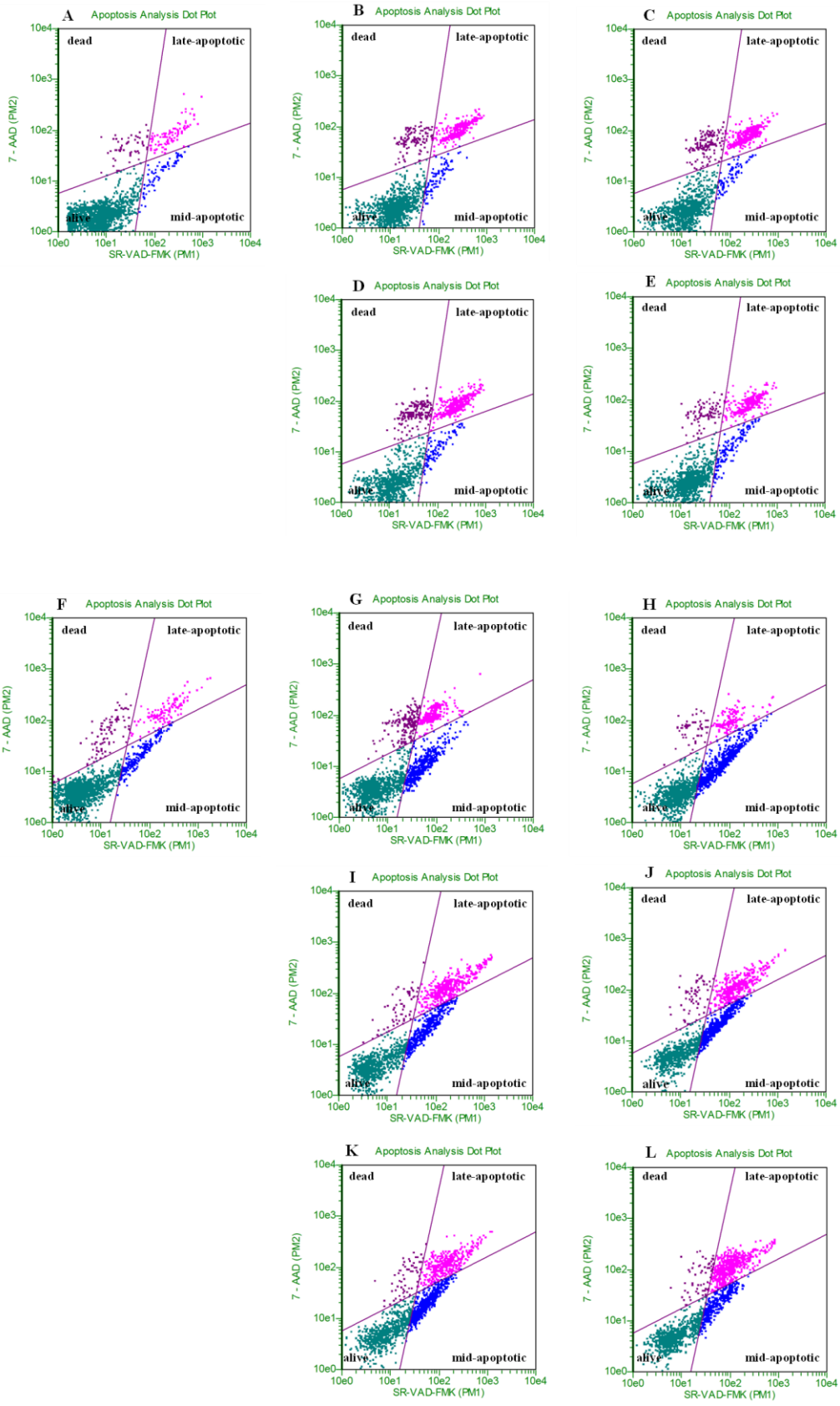
Results of Multi-caspase apoptotic assay, MTT assay and luciferase reporter array were expressed as means ± SD. ESI-MS detection, Multi-caspase apoptotic assay, MTT assay and luciferase reporter array were conducted in three separate experiments with three replicate. Confocal observations of annexin-V and cytochrome c were conducted in two separate experiments with duplicate. Statistical analysis was conducted using one-way ANOVA with Duncan post hoc comparison. Significance was determined at $p < 0.05$ using the SPSS statistical software (v12.0, Chicago, IL, USA).

7.3 Results

7.3.1 Response of Triterpenoids and the Alcohol Derivatives in Multi-caspase Apoptotic Assay

To quantify the stages of apoptosis and possible involvement of caspase cascade induced by *Ganoderma lucidum* triterpenoids and the alcohol derivatives in HeLa and Caco-2 cells, multi-caspase apoptotic assay was conducted and the results of the flow cytometry analysis are shown in **Figure 18** and **Table 13**. Compared to the controls, treatments of all tested samples are able to significantly ($p < 0.05$) reduce the percentage of viable cells from 10.51% to 35.67% and from 29.26% to 40.78% in Caco-2 and HeLa

cells respectively. Treatments of all six compounds also significantly ($p < 0.05$) increase cell percentages in mid and late-apoptotic stages in HeLa cells with increase ranges of 6.75% to 35.32% and 4.56% to 34.36% respectively. Similarly, treatments of ganodermanontriol, 7-oxo-ganoderic acid Z, 15-hydroxy-ganoderic acid S and ganoderic acid DM significantly ($p < 0.05$) stimulate late-apoptotic cells in Caco-2 cells with an increase range from 8.90% to 21.85%, while only 7-oxo-ganoderic acid Z and ganoderic acid DM are able to significantly ($p < 0.05$) stimulate mid-apoptotic cells in the same cell line with increase cell percentages of 5.48% and 11.68% respectively. Treatments of most of the compounds do not cause significant ($p < 0.05$) differences of percentage in dead cells except treatments of 7-oxo-ganoderic acid Z and 15-hydroxy-ganoderic acid S in Caco-2 cells which increase 3.5% and 5.3% respectively, and lucidumol A in HeLa cells which increases 7.95%. Among the six triterpenoids and the alcohol derivatives, ganoderic acid DM has the maximum reductions of viable cells and accumulations of late-apoptotic cells in both Caco-2 and HeLa cells. This triterpenoid also has the maximum cells accumulation in mid-apoptotic stage in Caco-2 cells compare to the other compounds but the cell percentage is less than that in late-apoptotic stage, indicating apoptosis induced by ganoderic acid DM is dominantly in late-apoptotic stage after 72 h treatment. Similar trends are shown in all treatments in Caco-2 cells and treatment of ganodermanontriol in HeLa cells. Conversely, ganolucidic acid E causes a maximum accumulation of mid-apoptotic cells and a minimum increase cell percentage in late-apoptotic stage in HeLa cells, suggesting apoptosis induced by ganolucidic acid E is dominantly in mid-apoptotic stage after 72 h treatment. Treatments of lucidumol A, 7-oxo-ganoderic acid Z and 15-hydroxy-ganoderic acid S follow the similar trend in HeLa cells.


A
Apoptosis Analysis Dot Plot
B
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K
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dead
late-apoptotic
alive
mid-apoptotic
7 - AAD (PM2)
SR-VAD-FMK (PM1)
10e0
10e1
10e2
10e3
10e4

Figure 18. Representative flow cytometry analysis of caspase activity of Caco-2 cells (A-E) and HeLa cells (F-J) treated with triterpenoids and the alcohol derivatives at their respective previously reported LC50 concentrations for 48 h. **A**, untreated control of Caco-2 cells; **B,** treatment of ganodermanontriol on Caco-2 cells; **C**, treatment of 7-oxo-ganoderic acid Z on Caco-2 cells; **D**, treatment of 15-hydroxy-ganoderic acid S on Caco-2 cells; **E**, treatment of ganoderic acid DM on Caco-2 cells; **F**, untreated control of HeLa cells; **G**, treatment of ganolucidic acid E on HeLa cells; **H**, treatment of lucidumol A on HeLa cells; **I,** treatment of ganodermanontriol on HeLa cells; **J**, treatment of 7-oxo-ganoderic acid Z on HeLa cells; **K,** treatment of 15-hydroxy-ganoderic acid S on HeLa cells; **L**, treatment of ganoderic acid DM on HeLa cells.

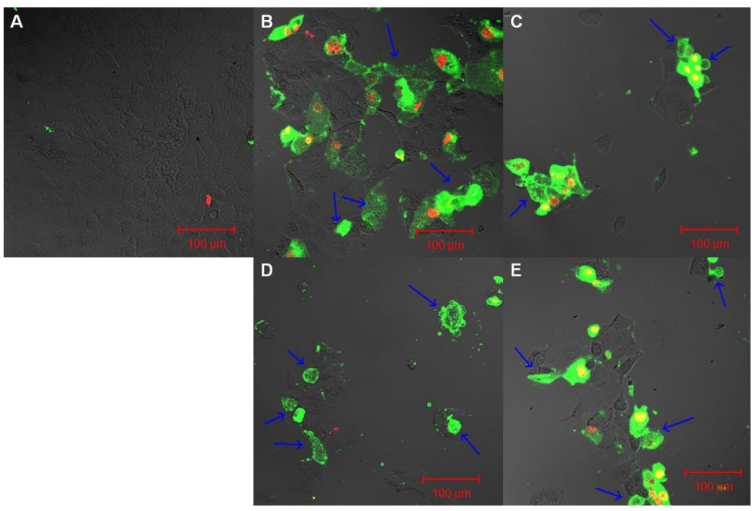
Table 13. Quadrant analysis of apoptotic stages by flow cytometry in multi-caspase apoptotic assay. An asterisk represents a significant difference ($p < 0.05$) compared to the corresponding control values.

Treatments on Caco-2 cells	Viable cells (%)	Mid-apoptotic cells (%)	Late-apoptosis cells (%)	Dead cells (%)
Control	83.33 ± 0.81	5.39 ± 0.46	6.52 ± 0.14	4.64 ± 0.46
Ganodermanontriol	72.82 ± 1.80*	5.471 ± 0.97	15.24 ± 2.01*	6.46 ± 0.21
7-oxo-ganoderic acid Z	58.63 ± 1.11*	10.87 ± 0.66*	22.34 ± 0.49*	8.14 ± 0.85*
15-hydroxy-ganoderic acid S	56.61 ± 2.84*	7.53 ± 0.61	25.92 ± 3.00*	9.94 ± 0.19*
ganoderic acid DM	47.66 ± 1.16*	17.07 ± 0.75*	28.37 ± 0.90*	6.91 ± 0.76
Treatments on HeLa cells				
Control	80.14 ± 1.10	10.34 ± 0.64	6.38 ± 054	3.03 ± 1.13
ganolucidic acid E	48.68 ± 2.55*	36.63 ± 1.31*	10.94 ± 1.59*	3.75 ± 0.40
lucidumol A	50.88 ± 1.38*	24.20 ± 0.30*	16.30 ± 0.80*	8.64 ± 0.69*
Ganodermanontriol	43.99 ± 2.31*	25.81 ± 0.90*	28.42 ± 2.14*	2.55 ±0.81
7-oxo-ganoderic acid Z	41.25 ± 0.64*	31.02 ± 2.63*	23.90 ± 1.75*	3.83 ± 0.42
15-hydroxy-ganoderic acid S	42.36 ± 1.28*	30.37 ± 2.21*	23.98 ± 1.16*	3.30 ± 0.32
ganoderic acid DM	39.36 ± 2.05*	17.09 ± 0.95*	40.74 ± 3.42*	2.79 ± 0.74

7.3.2 Translocation of Phophatidylserine

To determine the early apoptosis induced by *Ganoderma lucidum* triterpenoids and the alcohol derivatives in Caco-2 and HeLa cells, the translocations of phosphatidylserine were visualized by co-staining cells with annexin-V-FITC which specifically binds to

external cellular phosphatidylserine and PI which stains nuclear chromatin in cells without intact membranes. The results are shown in **Figure 19**. As negative controls, very little fluorescence signal is observed in Caco-2 and HeLa cells (**Figure 19 A &F**) without treatments. Conversely, green fluorescence from the annexin V-FITC conjugate is observed in all sample treatments, indicating all treatments are able to induce early apoptosis in Cao-2 and HeLa cells. It is also observed that this early apoptosis is dominant in treatments of 15-hydroxy-ganoderic acid S in Caoco-2 cells (**Figure 19 D**) and ganolucidic acid E, lucidumol A, ganodermanontriol, 15-hydroxy-ganoderic acid S and ganoderic acid DM in HeLa cells (**Figure 19 G-I, K&L**). Additionally, PI staining is observed to be positive for treatments of ganodermanontriol, 7-oxo-ganoderic acid Z and ganoderic acid DM in Caco-2 cells (**Figure 19 B, C & E**) and ganodermanontriol, 7-oxo-ganoderic acid Z, 15-hydroxy-ganoderic acid S and ganoderic acid DM in HeLa cells (**Figure 19 I-L**), indicating these treatments also results in late apoptotic and dead cells. Changes of morphology in apoptotic cells and fragments of nuclear chromatin are also observed in the sample treatments compared to the controls in both cell lines.



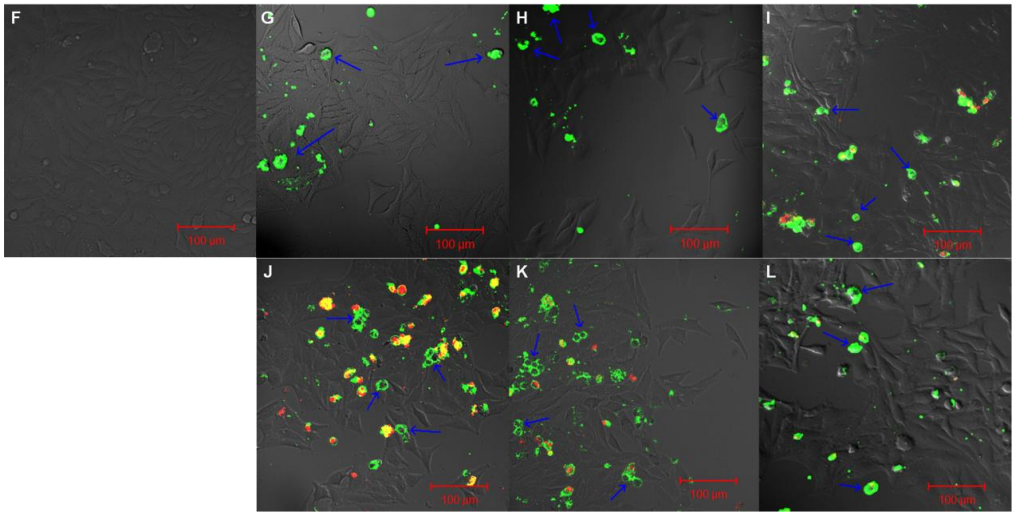
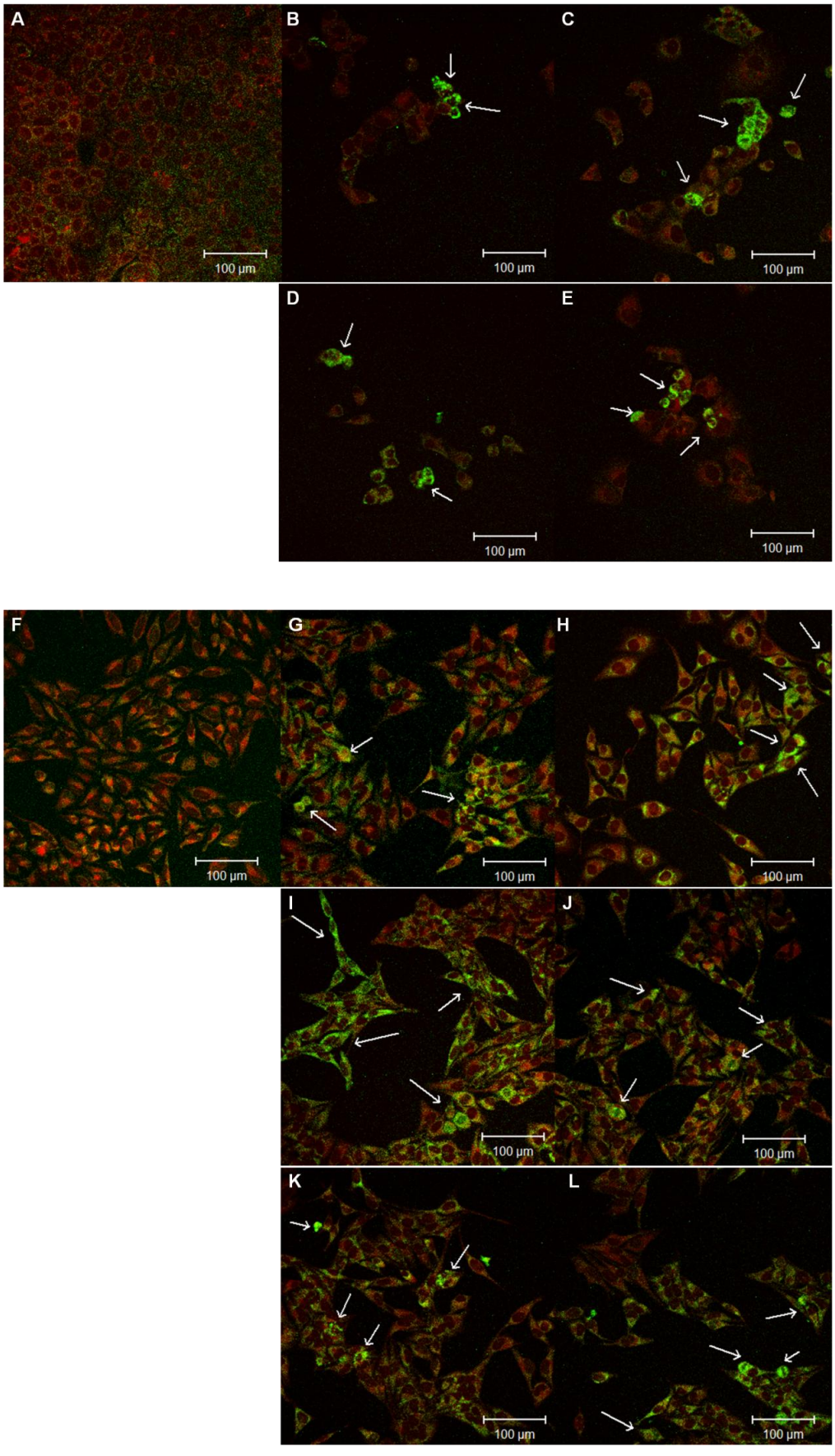


Figure 19. Confocal images of cells co-stained with annexin V-FITC with green signal and PI with red signal. Representative apoptotic cells were identified by the blue arrow. **A**, untreated control of Caco-2 cells; **B,** treatment of ganodermanontriol on Caco-2 cells; **C**, treatment of 7-oxo-ganoderic acid Z on Caco-2 cells; **D**, treatment of 15-hydroxy-ganoderic acid S on Caco-2 cells; **E**, treatment of ganoderic acid DM on Caco-2 cells; **F**, untreated control of HeLa cells; **G**, treatment of ganolucidic acid E on HeLa cells; **H**, treatment of lucidumol A on HeLa cells; **I,** treatment of ganodermanontriol on HeLa cells; **J**, treatment of 7-oxo-ganoderic acid Z on HeLa cells; **K,** treatment of 15-hydroxy-ganoderic acid S on HeLa cells; **L**, treatment of ganoderic acid DM on HeLa cells.

7.3.3 Observation of Cytochrome-c Reselase from Mitochondria

The visualizations of co-localization of cytochrome c and mitochondria and the release of cytochrome c by confocal microscope are shown in **Figure 20**. Cytochrome c in cells with treatments of all triterpenoids and alcohol derivatives are partially released into the cytosol from mitochondria in both Caco-2 and HeLa cells, which is evident by relatively strong green fluorescence from Anti-Cytochrome C FITC, compared to the co-localizations of cytochrome c and mitochondria in untreated control cells, which shows yellow or orange co-staining.


A
B
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E
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K
L
100 µm

Figure 20. Release of cytochrome c from mitochondria in response of apoptosis visualized by confocal fluorescence microscopy. Co-localization of cytochrome c in green signal and mitochondria in red signal were shown in **A**, untreated control of Caco-2 cells; **B,** treatment of ganodermanontriol on Caco-2 cells; **C**, treatment of 7-oxo-ganoderic acid Z on Caco-2 cells; **D**, treatment of 15-hydroxy-ganoderic acid S on Caco-2 cells; **E**, treatment of ganoderic acid DM on Caco-2 cells; **F**, untreated control of HeLa cells; **G**, treatment of ganolucidic acid E on HeLa cells; **H**, treatment of lucidumol A on HeLa cells; **I,** treatment of ganodermanontriol on HeLa cells; **J**, treatment of 7-oxo-ganoderic acid Z on HeLa cells; **K,** treatment of 15-hydroxy-ganoderic acid S on HeLa cells; **L**, treatment of ganoderic acid DM on HeLa cells. Representative cytochrome c releases were shown by the white arrow.

7.3.4 Signaling pathways involved in apoptosis induction

To determine possible pathways involved in the cellular regulation mediated by *Ganoderma lucidum* triterpenoids and the alcohol derivatives, six triterpenoids and alcohol derivatives, which were all able to induced apoptosis in HeLa cells, were tested using luciferase reporter arrays. Comparably, two of these triterpenoids exhibiting cytotoxicity but not inducing apoptosis, were also tested in Hep G2 cells as they represented the two major cell cycle arrest induction (S and G2/M) in Hep G2 as reported in chapter 6. The fold change of relative luciferase units compared to untreated controls of each pathway are summarized in **Figure 21**. Treatment of ganolucidic acid E results in significant ($p < 0.05$) up regulations of the reporter activity in Notch (RBP-Jκ reporter) (3.50 fold) and p53 pathways (2.08 fold) and down regulation of reporter activity in transforming growth factor beta (TGFβ, SMAD reporter) (0.73 fold), NFκB (0.20 fold), c-myc (myc reporter) (0.54 fold) and hypoxia (HIF reporter) pathways (0.53 fold) in HeLa cells. Treatments of lucidumol A causes significant ($p < 0.05$) increased associated-luciferase activity in p53 reporter (3.03 fold) and E2F reporter (cell cycle regulation pathway, 5.68 fold) while reduced the activity of NFκB (0.56 fold), myc (0.74 fold), HIF (0.49 fold), serum response element (SRE, MAPK/ERK pathway) (0.58 fold) and AP-1(MAPK/JNK pathway) (0.49 fold) reporters in HeLa cells. HeLa cells exposed to 7-oxo-ganoderic acid Z also results in significant ($p < 0.05$) up regulations of reporter

activity of RBP-Jκ (2.74 fold), p53 (2.62 fold) and E2F (2.26 fold) and down regulations of reporter activity of NFκB (0.75 fold) and HIF (0.78 fold). Exposure of the same cell line to 15-hydroxy-ganoderic acid S significantly ($p < 0.05$) up-regulates the activity of p53 (1.84 fold) and E2F reporter (1.80 fold) and down-regulates reporter activity of SMAD (0.33 fold), NFκB (0.66 fold) and myc (0.77 fold). Treatment of ganoderic acid DM causes similar up regulations in p53 (3.84 fold) and cell cycle signaling (2.83 fold) while it resulted in reduced activity of NFκB (0.75 fold), myc (0.73 fold) and HIF reporter (0.47 fold) in the same cell line. While treatment of ganodermanontriol did not result in any signaling up-regulation, instead the treatments significantly ($p < 0.05$) down-regulates the reporter activity of SMAD (0.43 fold) and NFκB (0.50 fold) in HeLa cells. Compared to treatments in HeLa cells, exposure of Hep G2 cells to the treatments of 15-hydroxy-ganoderic acid S resulted in significant ($p < 0.05$) up regulations in SMAD and E2F reporter activity with 2.87 and 2.45 fold respectively, while the reporter activity of Tcf/Lef (Wnt pathway), myc and AP-1 was significantly decreased by 0.69, 0.53 and 0.71 folds respectively. Treatments of ganoderic acid DM results in significant ($p < 0.05$) up regulations in reporter activity of RBP-Jκ (1.94 fold) and SMAD (2.81 fold) and down regulations in reporter activity of Tcf/Lef (0.75 fold) myc (0.53 fold) and HIF (0.42 fold). Neither of the treatments cause significant ($p < 0.05$) changes in p53, NFκB and SRE activity in HepG2 cells.

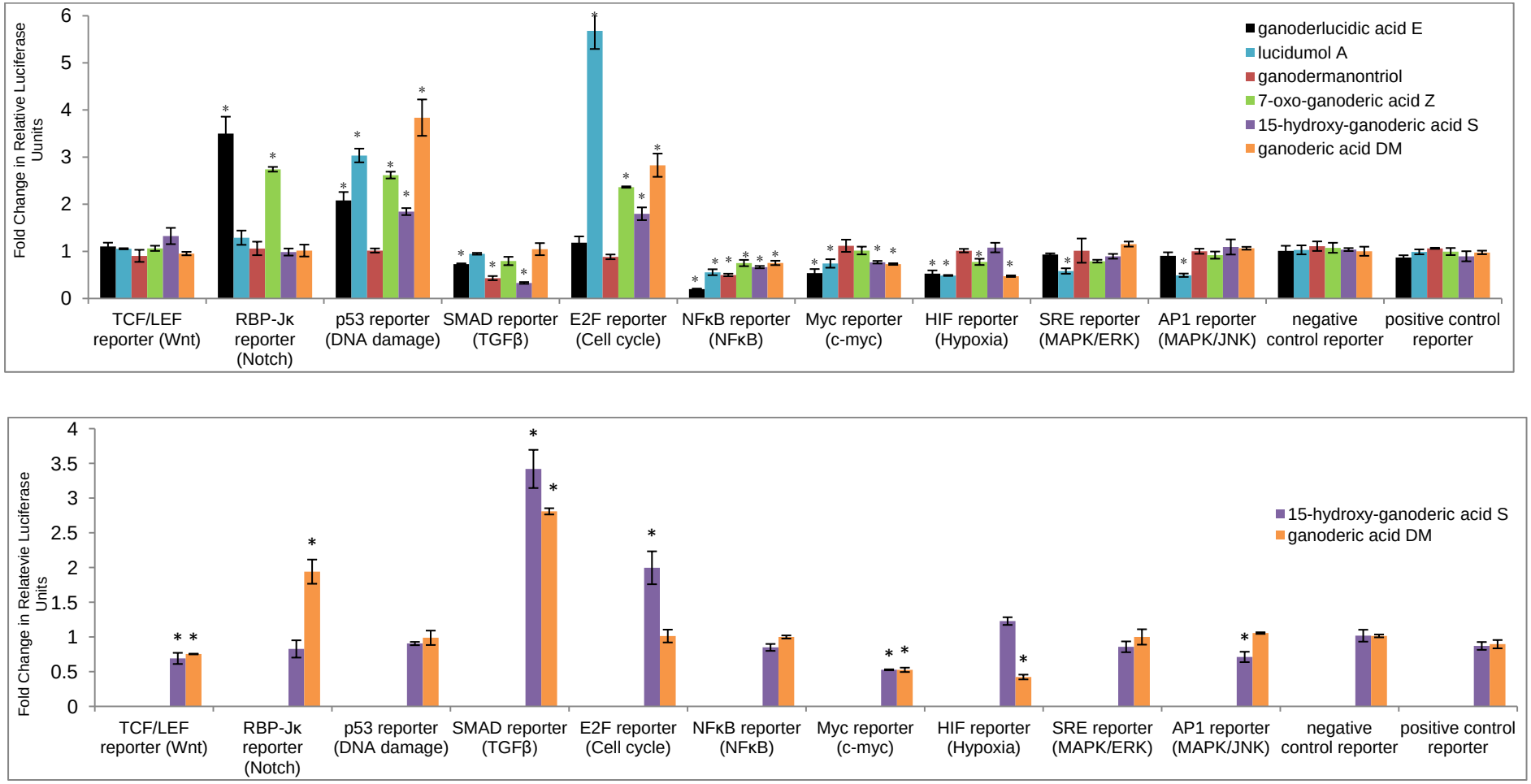

Figure 21. Signaling pathways in response to treatments of *Ganoderma lucidum* triterpenoids and alcohol derivatives in HeLa (upper panel) and Hep G2 cells (bottom panel). An asterisk represents a significant difference ($p < 0.05$) compared to the corresponding untreated control values.

7.4 Discussions

In chapter 6 we has reported that six triterpenoids and the alcohol derivatives induced apoptotic cell death and DNA fragmentation in HeLa cells and four of them had similar effects on Caco-2 cells by TUNEL assay. This chapter focuses on the possible mechanism of apoptotic cell deaths. The apoptotic cell deaths resulted from the six triterpenoids and alcohol derivatives were evidenced by the positive staining of multi-caspase activities using the fluorochrome conjugated caspase inhibitor of caspase 3, 7, 8 and 9 in Caco-2 and HeLa cell. Hep-G2 cell line was not used as we previously reported no induction of apoptosis after treatments in chapter 6. The percentages of cells at mid-apoptotic and late-apoptotic stages varied with respect to different sample treatments and cell lines in the multi-caspase apoptotic assay, which indicated that the apoptotic cell deaths induced by *Ganoderma lucidum* triterpenoids and the alcohol derivatives might be cell specific. However, no obvious structure-activity relationships regarding to this variations was observed. The implication of caspase-dependent apoptosis induced by *Ganoderma lucidum* triterpenoids and the alcohol derivatives were consistent with reported caspase activation of ganoderic acids in other carcinoma cells. For instant, caspase-3 but not caspase-8 was found to be stimulated during apoptosis induced by ganoderic acid T in 95-D cells [169]. Activation of caspase 3 was also observed in the apoptotic process with treatment of ganoderic acid Me in HCT-116 cells while caspase 3 and 9 were activated during apoptosis resulted from treatment of ganoderic acid Mf and S in HeLa cells [49;50]. However, the conclusion that *Ganoderma lucidum* triterpenoids and their derivatives induced apoptosis through activation of the same sets of caspase in different cell lines remains to be elucidated and requires further study.

It is well accepted that the apoptotic cell deaths are usually associated with externalization of phosphatidylserine from the inner leaflet of the plasma membrane into outer leaflet, which results from activation of calcium-dependent scramblase and inhibition of aminophospholipid translocase triggered by released cytochrome c from mitochondria [172]. The exposed phosphatidylserine in the surface of apoptotic cells subsequently signal the phagocytosis by recruiting specialized receptors of phagocytes for removal of apoptotic cells [173]. Detection of the externalization of phosphatidylserine and early stage of apoptosis are usually done by utilizing the fluorescent conjugate of Annexin V which exhibits strong and natural affinity to phosphatidylserine [174]. In this chapter, all six triterpenoids and alcohol derivatives showed positive results for phosphatidylserine externalization in HeLa cells and four in Caco-2 cells with various extents of annexin V-FITC and PI staining, indicating effects of triterpenoids and the alcohol derivatives also vary in the induction of the early stage of apoptosis. Results from the annexin V/PI apoptotic assay were consistent with the reported findings of treatments of ganoderic acid Me in HCT-116 cells, with more than 20% annexin V-positive apoptotic cells at 90.4 μM for 24 h treatment, and treatments of ganoderic acid Mf and S in HeLa cells at concentrations up to 97.7 μM, with a dose-dependent increase of early and late apoptotic cells [49;50].

Related to phosphatidylserine externalization, release of cytochrome c from mitochondria plays an important role in regulating apoptotic cell death. On one hand, cytochrome c released from mitochondria is thought to mediate oxidation of phosphatidylserine that subsequently interacts with aminophospholipid translocase and causes phosphatidylserine externalization [175]. On the other hand, the released cytochrome c can interact with apoptotic protease activating factor 1(Apaf1) and subsequent recruit caspase-9 forming apoptosomes [176]. In this chapter, the releases of

cytochrome c from mitochondria were observed in all sample treatments in Caco-2 and HeLa cells as expected, which further confirmed and supported the involvement of caspase activation and phosphatidylserine externalization during apoptosis induced by triterpenoids and the alcohol derivatives. The releases of cytochrome c were also consistent with reported observations in different cell lines with treatments of other *Ganoderma lucidum* triterpenoids, which include ganoderic acid T in 95-D cells with 3-fold increase in the cytochrome c level for 8 h treatment, ganoderic acid Mf and S in HeLa cells with up to 5.81-fold increase cytochrome c at a concentration of 97.7 μM and ganoderic acid Me in HCT-116 cells with 3-fold increase in the cytochrome c level for 12 h treatment at a concentration of 90.4 μM [49;50;169].

Multiple changes were observed in the luciferase reporter array after treatments with *Ganoderma lucidum* triterpenoids and the alcohol derivatives in both HeLa (apoptotic positive) and Hep G2 (apoptotic negative). Exposure of Hep G2 cells to 15-hydroxy-ganoderic acid S and ganoderic acid DM resulted in a significant decrease in the Wnt pathway reporter Tcf/Lef-associated luciferase activity while the activity of Tcf/Lef was not influenced by any of the treatments in HeLa cells. Aberrant activation of Wnt-signaling, which involves stabilization and accumulation of β-catenin that interact with Tcf/Lef to initiate transcription of proliferation stimulating genes such as c-myc and cyclin D1, has been reported to closely related to the progression of human cancers including colorectal cancer and hepatocellular carcinoma [177]. Inhibition of Wnt-signaling was found to inhibit cell growth and induce apoptosis in carcinoma cells [69;178]. In particular, the *Ganoderma lucidum* alcohol, ganodermanontriol has been reported to inhibit transcription activity of β-catenin, down-regulate cyclin D1 and inhibit cell growth in HT-29 colon carcinoma cells, which indicated an involvement of Wnt-signaling [69]. However, ganodermanotriol did not participate in the Wnt-signaling

in HeLa cells based on our analysis, indicating the Wnt-signaling regulation may be cell specific and the detailed regulation of the Wnt family proteins may need further exploration.

The luciferase reporter array indicated that the associated-luciferase activity of the Notch reporter, RBP-Jκ (also known as C protein binding factor 1/Suppressor of Hairless/Lag1 (CBF1/ Su (H)/Lag 1)), was significantly up-regulated by ganolucidic acid E and 7-oxo-ganoderic acid Z in HeLa cells, and ganoderic acid DM in Hep G2 cells. The Notch pathway has been implicated in mammary carcinogenesis but its role in carcinogenesis was reported to be controversial and probably to be highly cell and context specific. This is likely due to the complex interplay of four receptors (Notch1, Notch 2, Notch 3 and Notch4) and five ligands (Jagged1, Jagged2, Delta-like ligand 1, ligand 3, and ligand 4) in this signaling and the crosstalk of Notch signaling with NF-κB, Akt and P53 signaling [179-183]. Detailed examinations of the activity in four receptors are required to elucidate impacts of *Ganoderma lucidum* triterpenoids and the alcohol derivatives on Notch signaling in the two cell lines in the future.

As the well accepted tumor-suppressor gene, the associated-luciferase activity of p53 was observed to significantly increase in the treatments of ganolucidic acid E, lucidumol A, 7-oxo-ganoderic acid Z, 15-hydroxy-ganoderic acid S and ganoderic acid DM in HeLa cells but not with treatments of ganodermanontriol in HeLa cells or 15-hydroxy-ganodric acid S and ganoderic acid DM in Hep G2 cells. The increased p53 reporter activity reported in this chapter is consistent with data in the previous chapter (chapter 6), which showed significant increase in DNA fragmentation in TUNEL assay in Caco-2 and Hela cells. In the previous chapter (chapter 6), however, the cell percentage with DNA fragmentation of ganodermanontriol treatment in HeLa cells had a minor increase though it had statistical differences compared to control cells and concomitantly, the p53 reporter

activity was not activated by ganodermanontriol in this chapter. Similarly, the inability to stimulate p53 reporter activity in Hep G2 cells is consistent with the negative results of zero sub-G1 accumulation in cell cycle analysis in chapter 6. The results in this chapter are also consistent with the up-regulation of p53 expression as reported in the treatments of ganoderic acid Me in multidrug resistant clone cancer cells and HCT-116 cells and ganoderic acid T in 95-D cells [49;169;184].

The TGF β signaling has been suggested biphasic stage-specific effects in carcinoma development. On one hand, down regulation and inactivation of TGF β receptors and SMADs have been implicated in the development of various human cancers. Therefore, TGF β served as tumor suppressor in epithelial cell growth by inducing apoptosis and modulating cell cycle regulators in the stage of carcinoma initiation [185;186]. On the other hand, high level expression of TGF β was found to be associated with progressive cell proliferation, invasion and metastasis in the advanced staged of established tumors [187;188]. *Ganoderma lucidum* has been reported to inhibits prostate cancer-dependent angiogenesis with decreased secretion of vascular endothelial growth factor and TGF β1 from PC-3 highly invasive human prostate cancer cells [189]. Consistent with this report, exposure of ganolucidic acid E, ganodermanontriol and 15-hydroxy-ganoderic acid S to HeLa cells resulted in a significant reduction in the associated luciferase activity of TGF β reporter SMAD. Conversely, significant activation of SMAD was observed in treatments of 15-hydroxy-ganoderic acid S and ganoderic acid DM in Hep G2 cells. Results suggested that TGF β may regulate differently in response to *Ganoderma lucidum* triterpenoids and the alcohol derivatives mediated- cell growth inhibition in HeLa and Hep G2 cells.

In chapter 6, cell cycle arrest has been observed in *Ganoderma lucidum* triterpenoids and the alcohol treatments in Caco-2, HeLa and Hep G2 cells. In this study, the associated

luciferase activity of E2F which is a reporter related to cell cycle progression, was observed to significantly increase in the treatments of lucidumol A, 7-oxo-ganoderic acid Z, 15-hydroxy-ganoderic acid S and ganoderic acid DM in HeLa cells and 15-hydroxy-ganoderic acid S in Hep G2 cells. Conversely, exposure of HeLa cells to ganolucidic acid E, lucidumol A, 15-hydroxy-ganoderic acid S and ganoderic acid DM and exposure of Hep G2 cells to 15-hydroxy-ganoderic acid S and ganoderic acid DM all resulted in significant reduction in the associated-luciferase activity of myc, which was another reporter related to cell cycle progression. Similar results have been reported that increased expression of E2F correlated to suppression of cell proliferation in HeLa and Hep G2 cells [190;191]. Our findings were also consistent with the reports that down regulation of myc were associated with cell proliferation inhibition and apoptosis inductions in HeLa and Hep G2 cells [66;192]. However, the E2F and myc families of transcription factors both have been reported to possess the dual functions in transactivate and repress gene expression that regulate DNA replication, mitosis, DNA-damage and repair, apoptosis and differentiation [193-195]. It is likely that these two reporters mediate the *Ganoderma lucidum* triterpenoids and the alcohol derivatives induced-cell cycle arrest and apoptosis independently or through interacting with other transcription factors such as SMADs and Tcf/Lef, which need further investigations on their upstream and downstream signaling.

Apart from induction of cell cycle arrest and apoptosis, *Ganoderma lucidum* extract and its triterpenoids have been reported to inhibit metastasis and invasion in MCF-7 and Hep G2 cells through inhibition of phosphorylation of ERK1/2 and reducing AP-1 and NF-κB [196;197]. Consistent with these reporting signaling, the associated luciferase activity of NF-κB was found to be significantly decreased with treatments of all six triterpenoids and the alcohol derivatives in HeLa cell, while its activity in Hep G2 cells

were not affected. Activity of AP-1 was significantly reduced with exposure to lucidumol A in HeLa cells and 15-hydroxy-ganoderic acid S in Hep G2 cells. The activity of SRE, which is the ERK1/2 reporter, was observed to significantly decrease with exposure to lucidumol A in HeLa cells. Reduced level of these reporters may play a role in the inhibition of transactivation of cyclin D1, Bcl-2, COX-2 and activation of p16, p21 and p53, which closely regulate cell cycle progression, cell proliferation, invasion and metastasis [198-201].

In this chapter, we also found that the associated-luciferase activity of HIF, the key transcription factor of hypoxia, was significantly reduced by treatments of ganolucidic acid E, lucidumol A, 7-oxo-ganoderic acid Z and ganoderic acid DM in HeLa cells and ganoderic acid in Hep G2 cells. Hypoxia has been reported to trigger tumor angiogenesis and correlate positively to tumor malignant progression, chemoradio-resistance and poor prognosis. Studies have indicated that activation of HIF was responsible for a number of oncogenic signaling activation and tumor suppressors inactivation [202;203]. This is the first report indicating that suppression of HIF might be associated with the regulation of cell proliferation and apoptosis mediated by *Ganoderma lucidum* triterpenoids and the alcohol derivatives in HeLa and Hep G2 cell.

Taken together, the apoptotic cell deaths triggered by treatments of *Ganoderma lucidum* triterpenoids and the alcohol derivatives were associated with caspase activation, phosphatidylserine externalization and cytochrome c release. The apoptosis was also suggested to involve multiple signaling mechanisms in HeLa cells. However, it is not yet clear which signaling would be the direct targets of these triterpenoids and the alcohol derivatives and whether indirect signaling regulations could be involved. Further investigation may be needed to look into the involving regulation signaling and their possible interactions.

CHAPTER 8 OVERALL CONCLUSIONS AND FUTURE STUDY

In this thesis, the chemical characterizations of bioactive components of *Ganoderma lucidum* have been conducted and methods were developed to isolate and identify *Ganoderma lucidum* lipids and triterpenoids and their derivatives. *Ganoderma lucidum* lipids were found to consist of long chain fatty acids, hydroxyl fatty acids and possibly sterol derivatives and phospholipids while the exact structures of some lipid components remained unknown (Chapter 4). Further isolation and identification of these components are required in the future. In addition to *Ganoderma lucidum* lipids, a total of 21 triterpenoids and derivatives have been identified in *Ganoderma lucidum* and 13 of them have been isolated as pure compounds from two triterpenoid enriched extracts (Chapter 3, 5 and 6). Extraction conditions such as extraction time and the ratio of organic solvents were found to significantly influence the extraction yield of triterpenoids and optimal extraction conditions of certain triterpenoid have been developed using a quadratic model (Chapter 5). Future work should be centered on triterpenoids and their derivatives that have not been adequately identified and isolated from *Ganoderma lucidum.*

On the other hand, *Ganoderma lucidum* lipids and triterpenoids and their derivatives have been well documented in this thesis to possess possible chemo-preventative properties with diverse bioactive effects on three cultured human carcinoma cells. Initial observations have showed that two triterpenoids fractions (fraction 2 and 3) separated from an ethanol extract of *Ganoderma lucidum* were able to reduce the Caoc-2 cell viability dose dependently and cause a cell cycle arrest in G2/M phase, but only one of them (fraction 3) induced apoptotic cell death in Caco-2. The results indicated that variation in polarity of triterpenoids in these extracts may specifically influence the cyto-response in the cells (Chapter 3).

The subsequent investigations have extended the initial understating to the bioactivity of 13 triterpenoids and the alcohol derivatives in Caco-2, HeLa and Hep G2 cells. An implication of potential structure-activity relationships have been established that triterpenoids without functional group at C-23 position and less saturation of the side chain could result in greater cytotoxicity in carcinoma cells (Chapter 3, 5 and 6). Moreover, the effects of 6 cytotoxic triterpenoids and the alcohol derivatives in three cell lines were shown to be cell-specific and dependent on structure, which is evident by the variation of cytotoxicity and cell cycle arrest at G1, S and G2/M phase induced by different compound treatments in Caco-2, HeLa and Hep G2 cells (Chapter 6). The apoptosis response also varied with respect to the six triterpenoids and alcohol derivatives, all of which had different extents of sub-G1 accumulation and positive staining in TUNEL assay in HeLa cells and four in Caco-2 cells but none in Hep G2 cells (Chapter 6).

The apoptotic cell death induced by cytotoxic triterpenoids and the alcohol derivatives in Caco-2 and HeLa cells were associated with caspase activations, phosphatidylserine externalization and cytochrome c release as detected by multi-caspase apoptotic assay, annexin-V-FITC/PI staining and anti-cytochrome c-FITC staining respectively (Chapter 7). A screening of possible pathways for apoptosis regulation suggested that multiple signaling pathways could be involved in the triterpenoids and alcohol derivatives mediated-apoptosis in HeLa cells, most of which include transcription activation of p53 and E2F and down regulation of NFκB, Myc and HIF (Chapter 7). Since some of signaling could crosstalk and interact with one another, future studies should centre on the upstream and downstream signaling of each pathway. Elucidation of the direct target of these signaling regulations would aid in understanding of the

structure-activity relationship of *Ganoderma lucidum* triterpenoids and the derivatives as apoptosis inducer in chemotherapy.

The bioactivities of a lipid enriched fraction and its ethanol crude extract of *Ganoderma lucidum* were also conducted in Caco-2 cells. The ethanol crude extract which contained triterpenoids besides lipids were found to be cytotoxic to Caco-2 cells as expected but its cytotoxicity rapidly declined after cells reaching confluency. Unlike triterpenoids from *Ganoderma lucidum*, the lipid enriched extract did not exhibit cytotoxicity to Caco-2 cells. Neither of these extracts induced apoptosis in Caco-2 cells but instead, they induced differentiation in Caco-2 cells with significant stimulation of alkaline phosphates and lactase activity (Chapter 4). Further analysis indicated that the differentiation induced was associated with a G2/M cell cycle arrest and possibly mediated through activation of JNK and ERK pathways and an indirect activation of PKC (Chapter 4). Future study is needed to examine the involved pathway signaling at posttranscriptional level is proposed to elucidate the exact underlying mechanisms. Nevertheless, the capability of *Ganoderma lucidum* lipids to induce differentiation in colonic cells give it potential as therapeutic agents for restoring carcinoma cells into normal cell and controlling cell growth.

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APPENDIX 1

1. Published Papers

(1) Ruan, W.; Popovich, D. G. Evidence of bioactivity from *Ganoderma lucidum* triterpenoids in cultured cell models. In *Saponins: Properties, Applications and Health Benefits*, Nova Science Publishers. **2011**, 145-156.

(2) Ruan, W.; Popovich, D. G. *Ganoderma lucidum* triterpenoid extract induces apoptosis in human colon carcinoma cells (Caco-2). *Biomedicine and Preventive Nutrition*. **2012**, 2 (3), 203-209.

2. Manuscripts Ready to be Submitted

(1) Ruan, W.; Popovich, D. G. *Ganoderma lucidum* extract and its lipid enriched fraction induce differentiation in Caco-2 human colon carcinoma cells. *xxx*. **2013**.

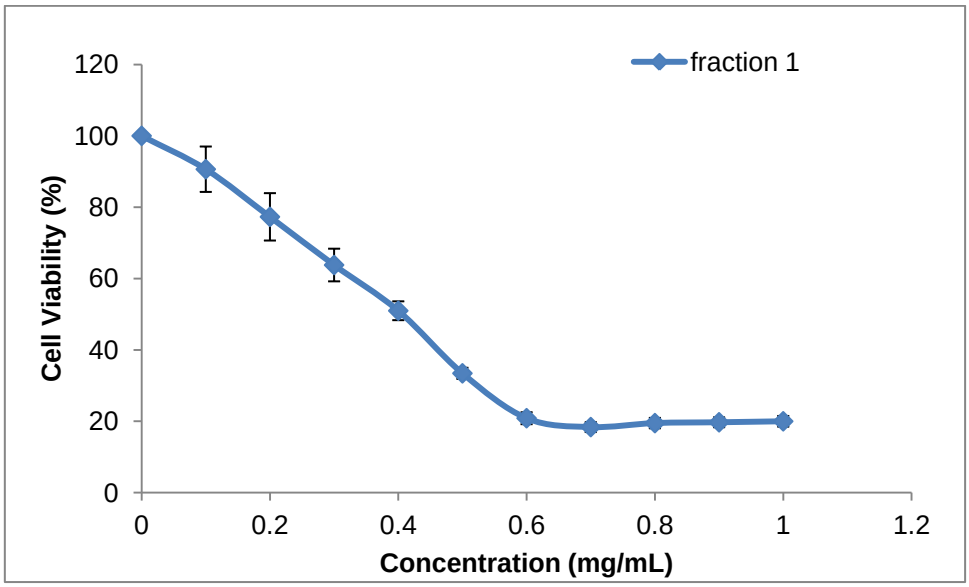
(2) Ruan, W.; Lim H.H.A.; Lau G.H.; Popovich, D. G. Extraction optimization and isolation of triterpenoids from *Ganoderma lucidum* and their cytotoxicities to cultured human carcinoma cells. *xxx*. **2013**.

(3) Ruan, W.; Popovich, D. G. Cytotoxic triterpenoids and alcohol derivatives from *Ganoderma lucidum* show various effects on cell cycle distribution and apoptosis in three cultured human carcinoma cells. *xxx*. **2013**.

(4) Ruan, W.; Wei, Y.; Popovich, D. G. *Ganoderma lucidum* triterpenoids and the alcohol derivatives stimulate caspase activation and cytochrome c release, induce apoptosis and alter multiple cellular pathways in cultured human carcinoma cells. *xxx*. **2013**.

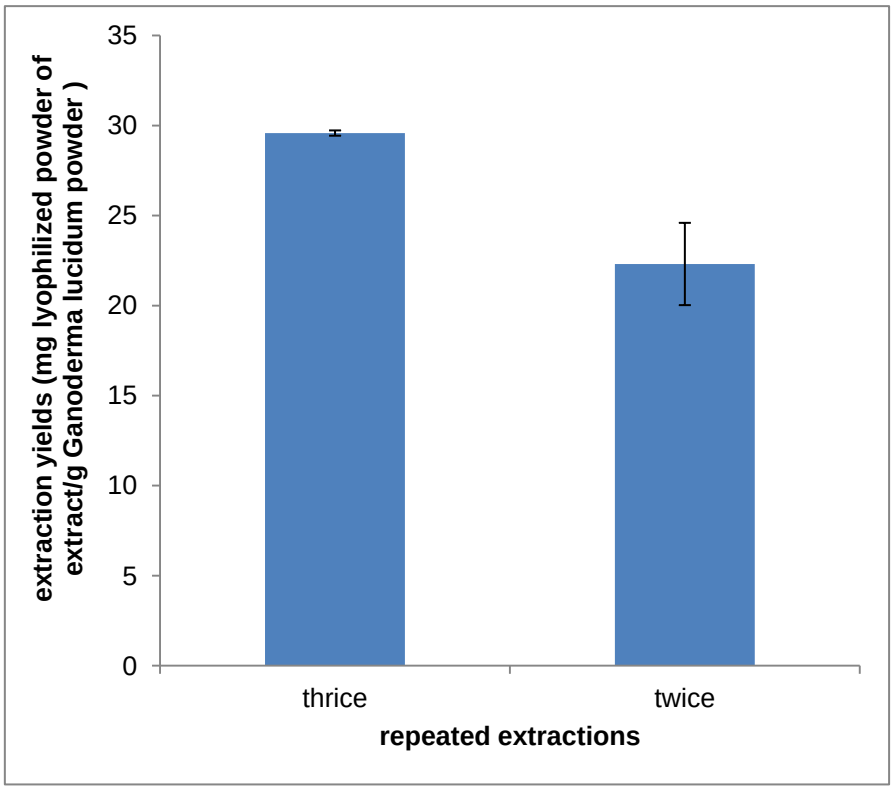
APPENDIX 2

Cell viability of fraction1 treated Caco-2 cells for 72h and assessed using the MTT assay. Values are expressed as mean ± SD (percentage of untreated control cells) of three separate experiments with three replicate.



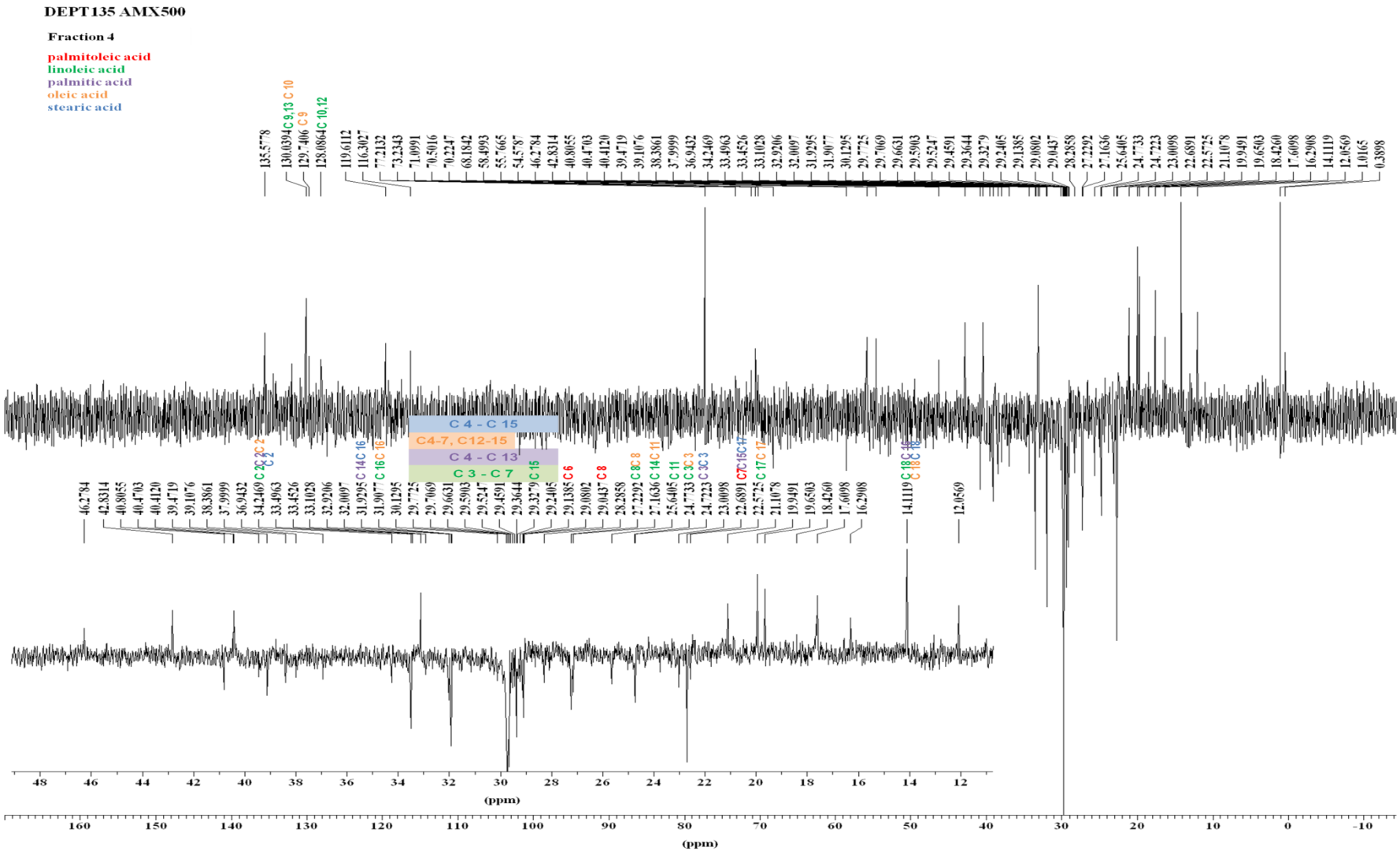
APPENDIX 3

Effects of repeated extractions on extraction efficiency of *Ganoderma lucidum* triterpenoids. Values are expressed as mean ± SD (mg lyophilized powder of extract/g *Ganoderma lucidum* powder) of three replicate experiments. Reflux extraction were applied with 100% ethanol for 3 hr.



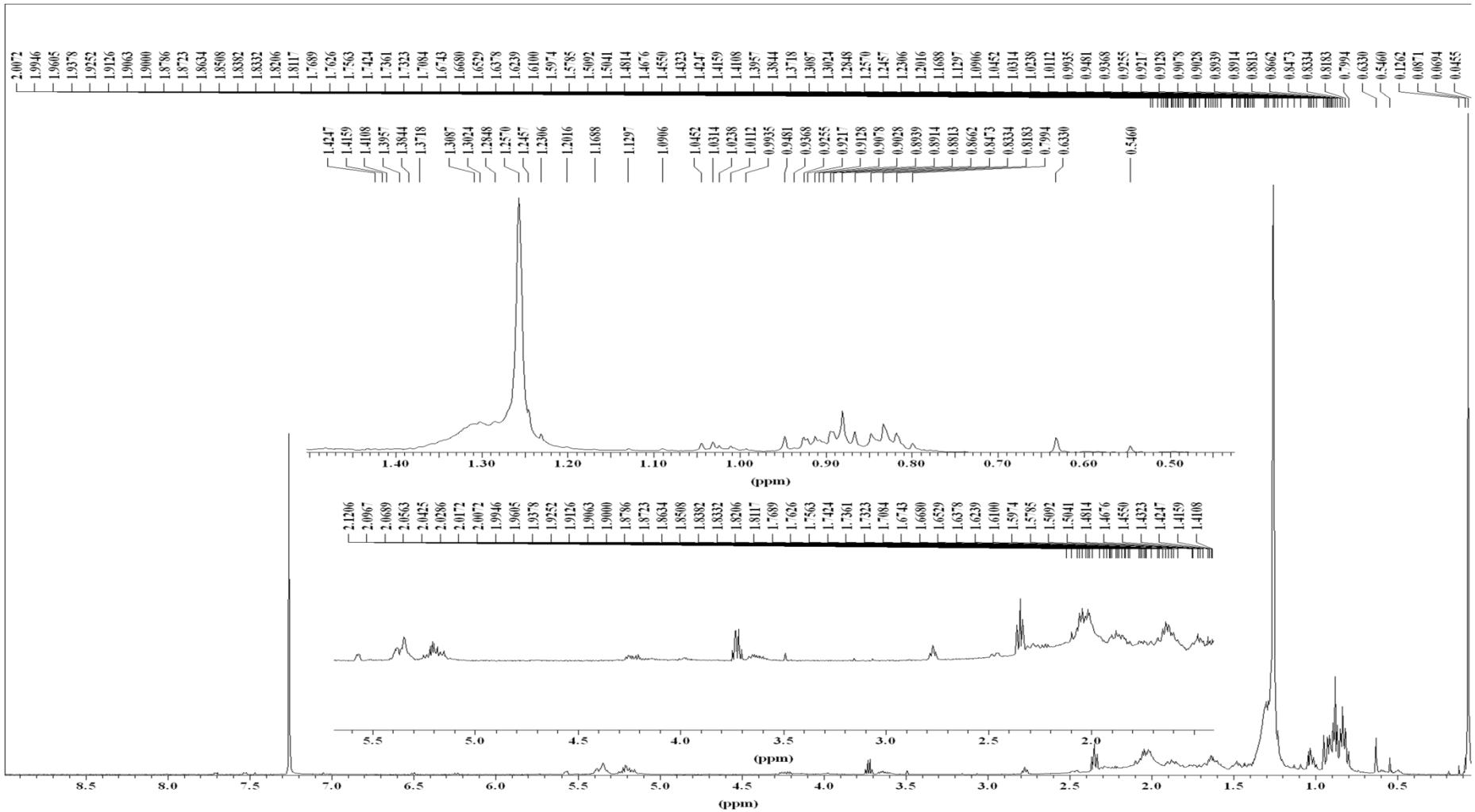
APPENDIX 4

DEPT ^{13}C and ^{1}H NMR spectrum of fraction 4 from *Ganoderma lucidum* extraction in this study


DEPT135 AMX500
Fraction 4
palmitoleic acid
linoleic acid
palmitic acid
oleic acid
stearic acid
C 4 - C 15
C4-7, C12-15
C 4 - C 13
C 3 - C 7
(ppm)
(ppm)

1H AMX500

Fraction 4

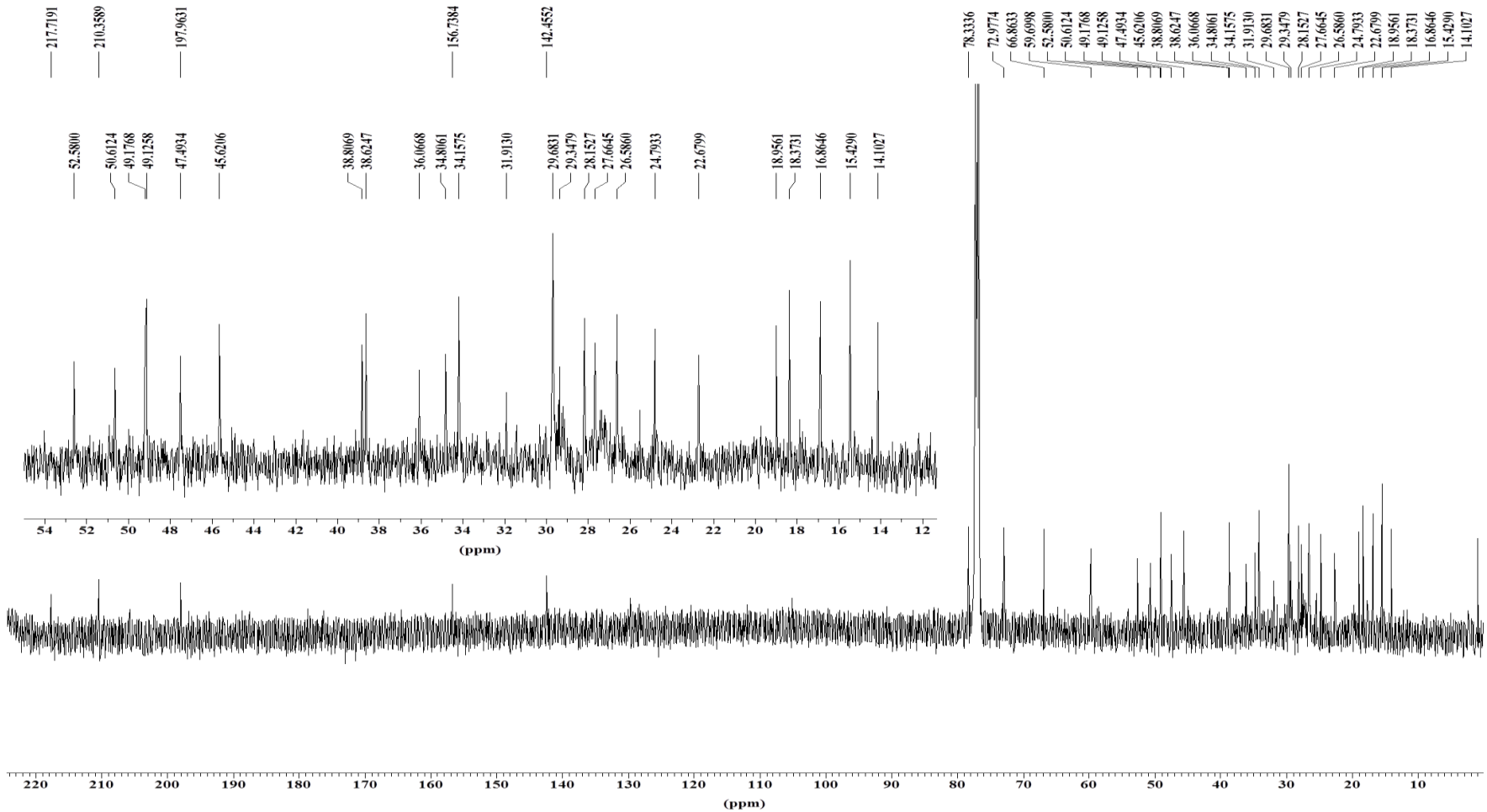


APPENDIX 5

^{13}C and DEPT ^{13}C NMR spectrum of identified triterpenoids and alcohol derivaties from *Ganoderma lucidum* in this study

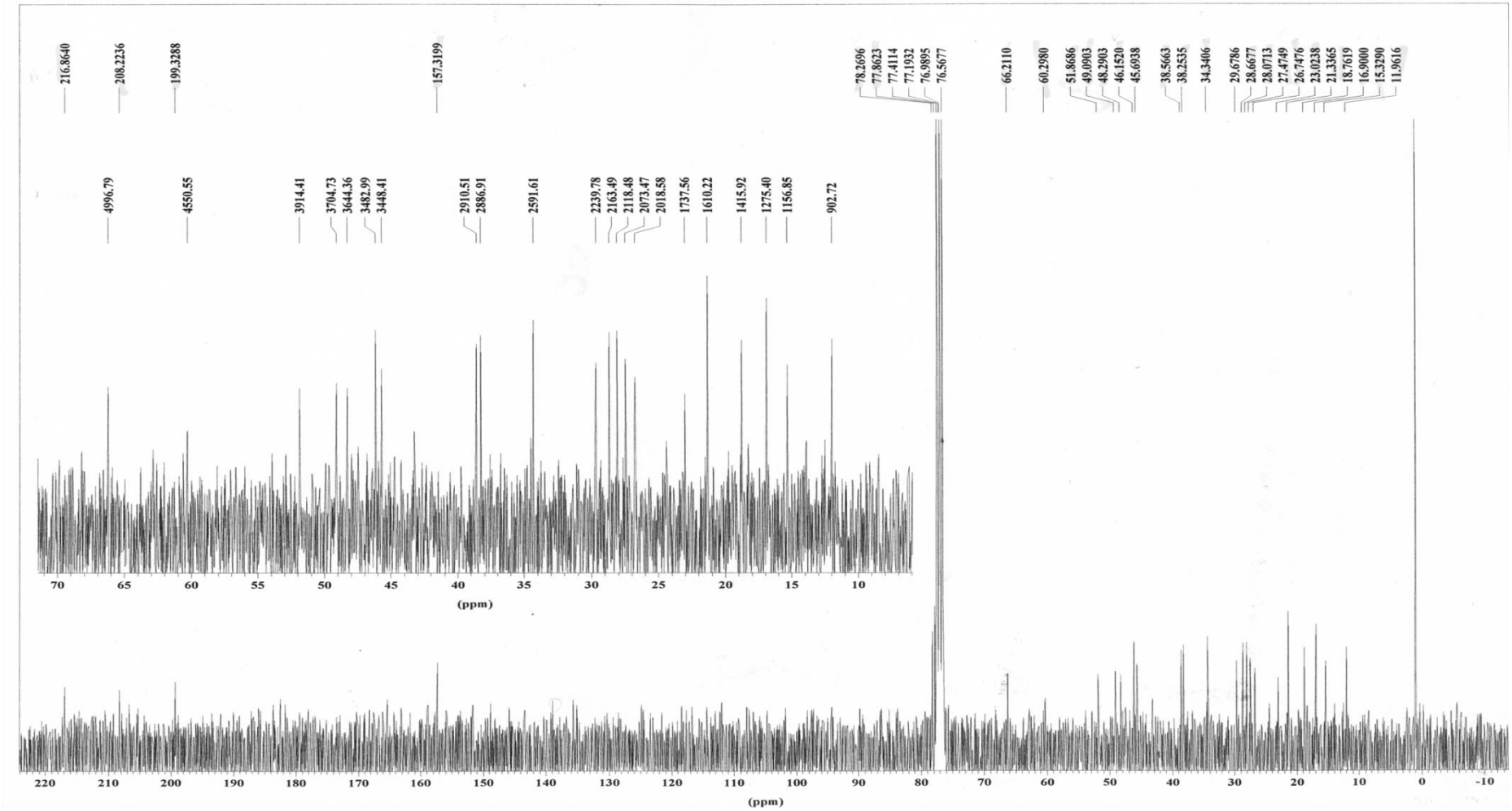
13C AMX500

Ganoderic acid I



13C Standard AC300

Ganoderic acid G



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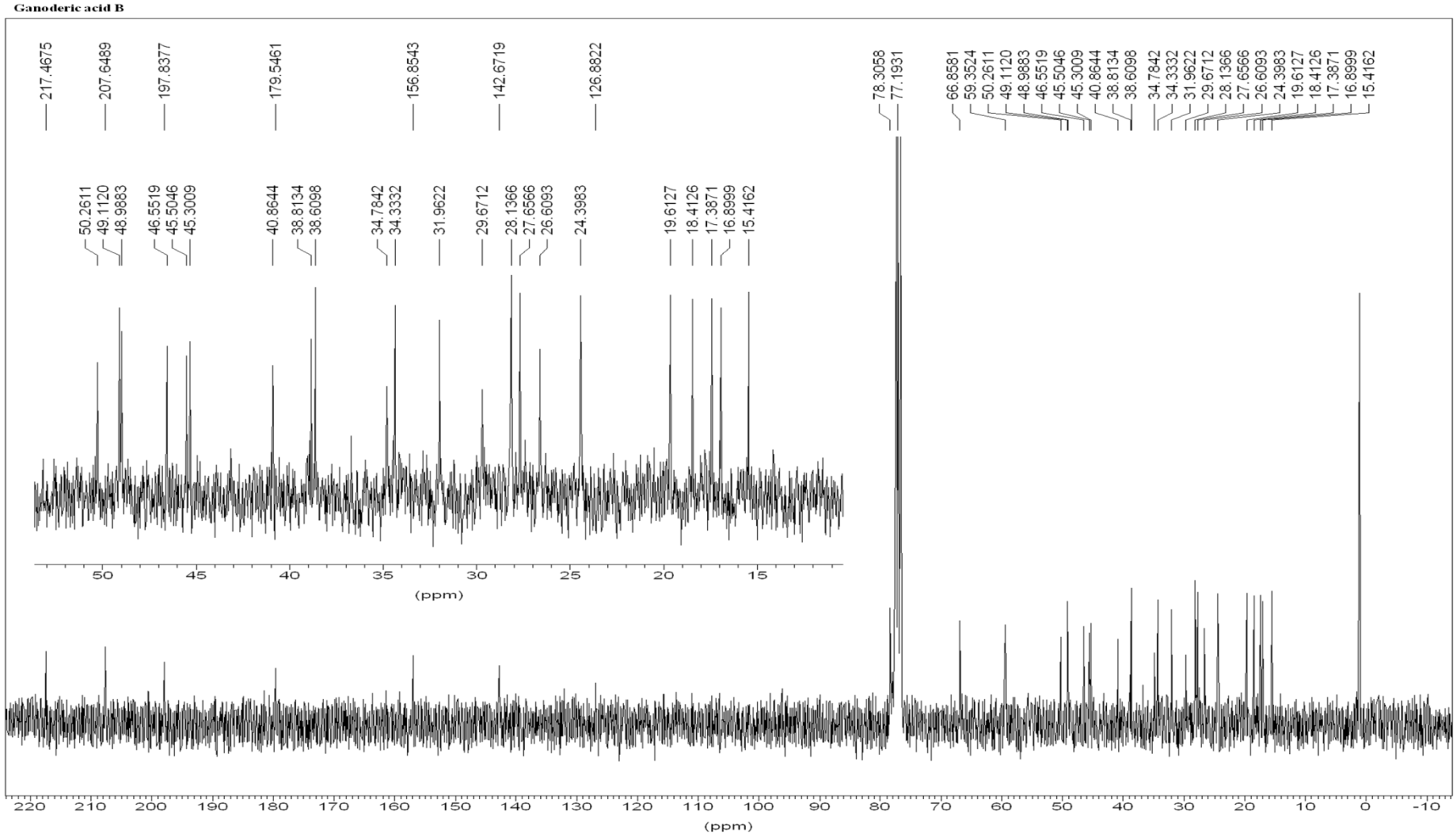
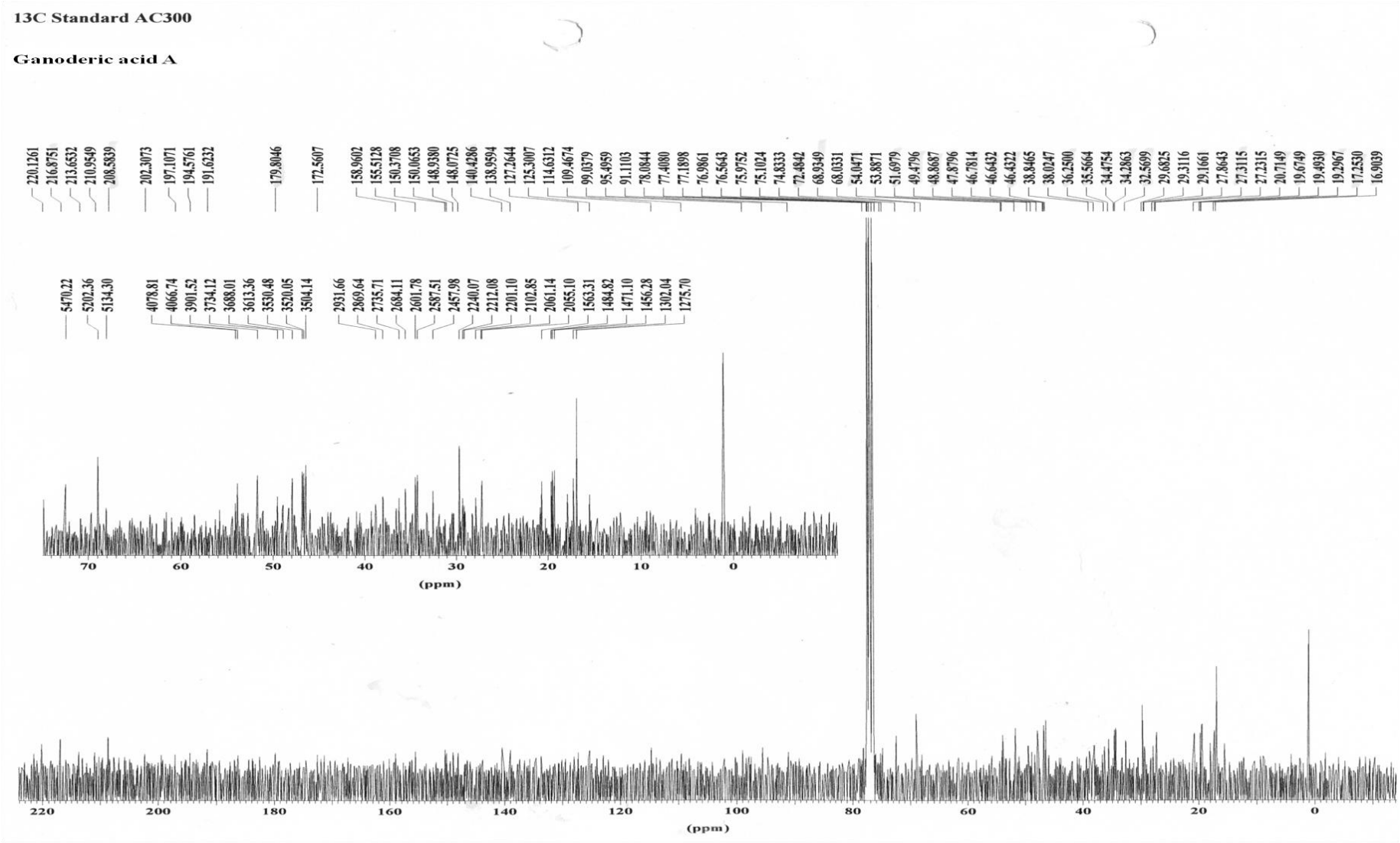


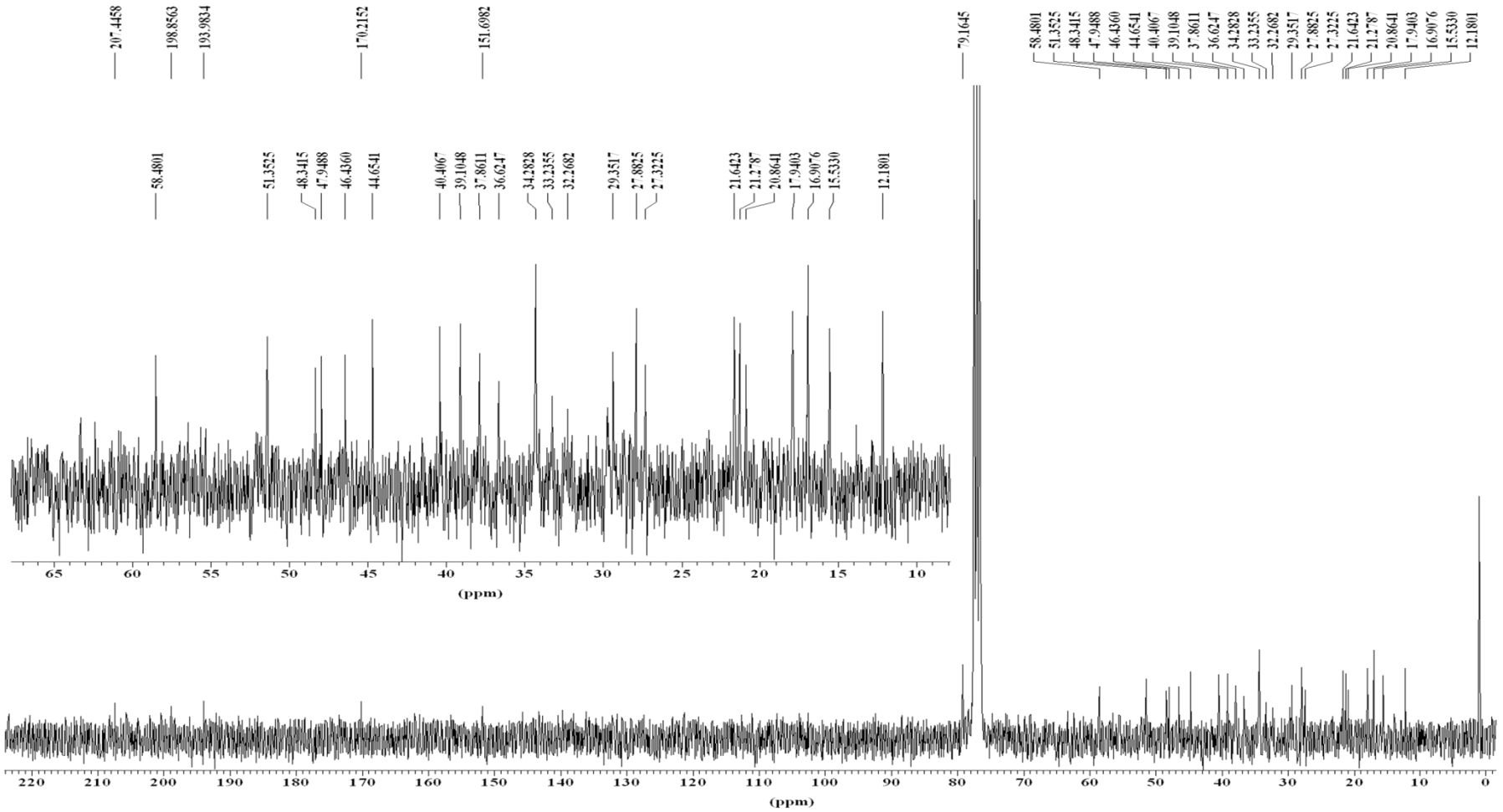
13C Standard AC300

Ganoderic acid A



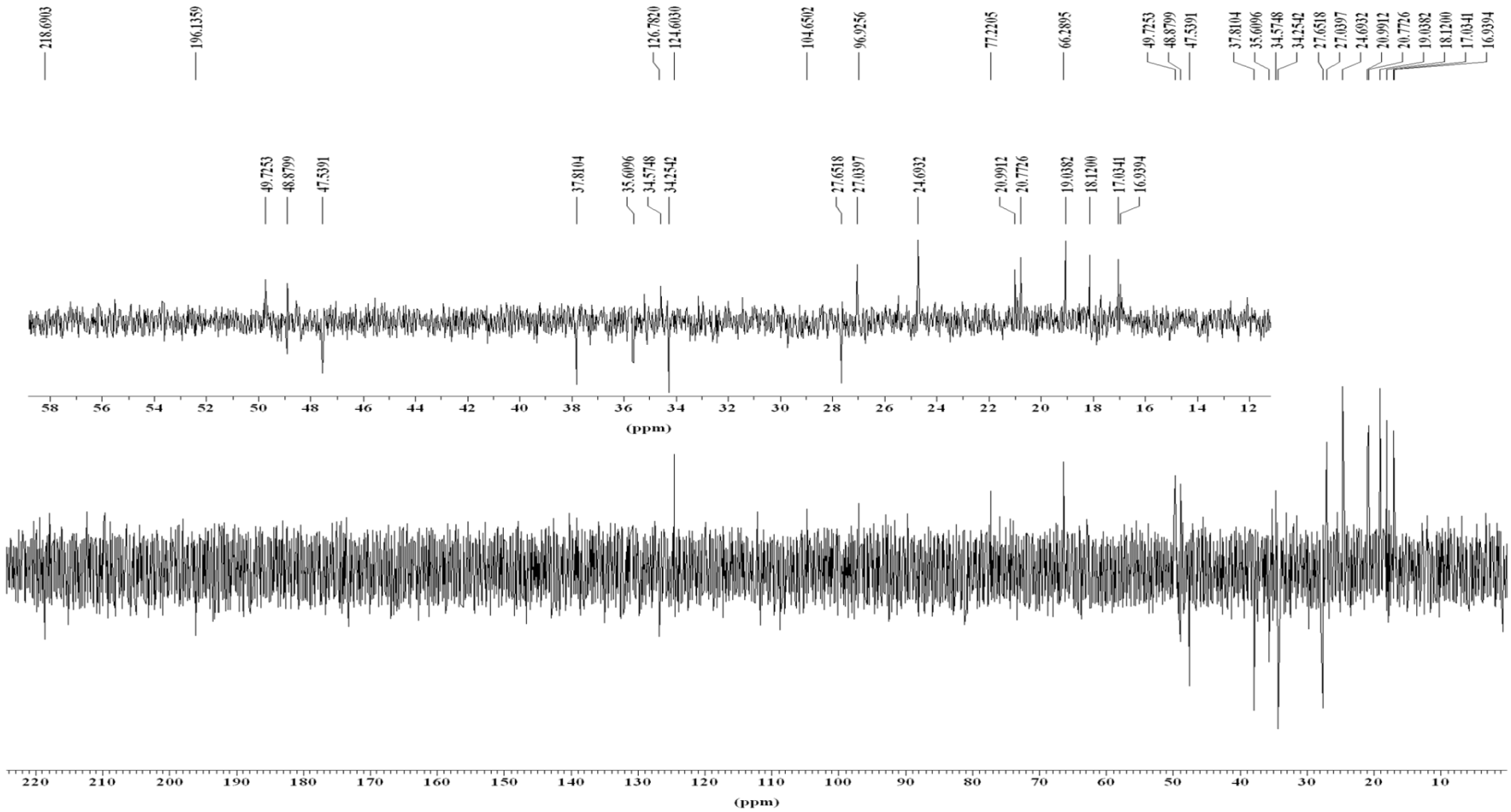
13C Standard AC300

Ganoderic acid H



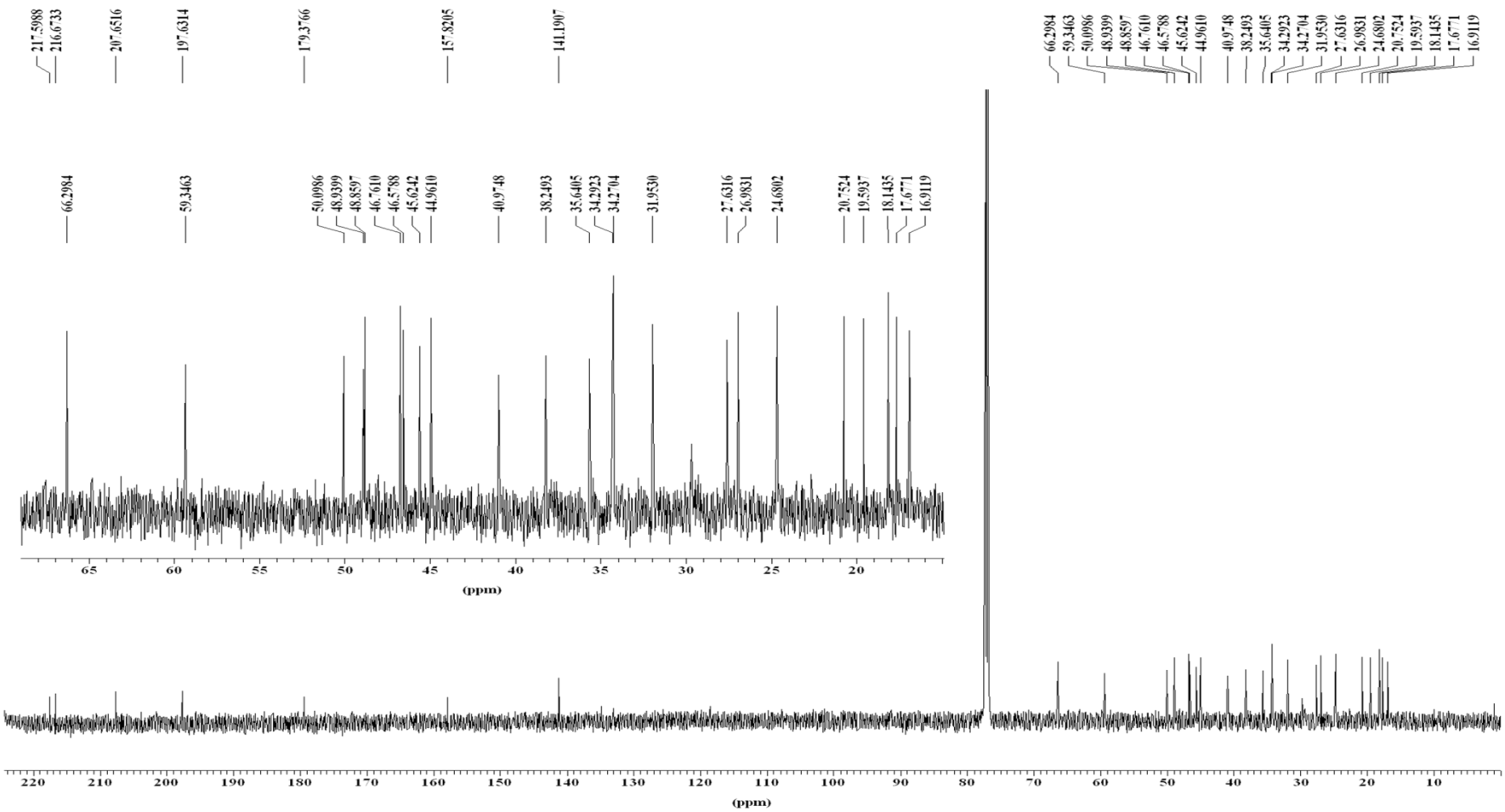
DEPT135 AMX500

Ganoderenic acid D



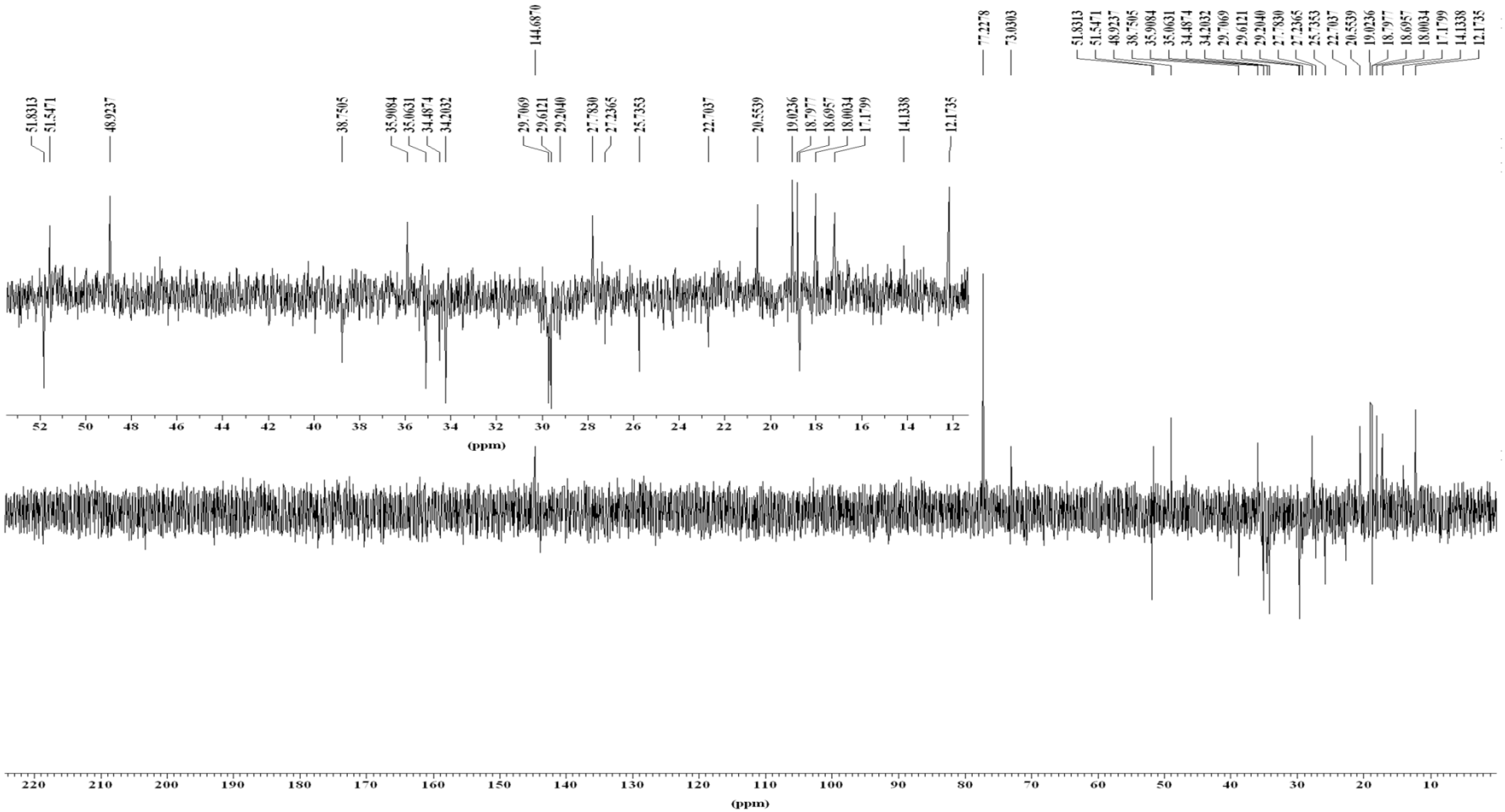
13C AMX500

Ganoderic acid D



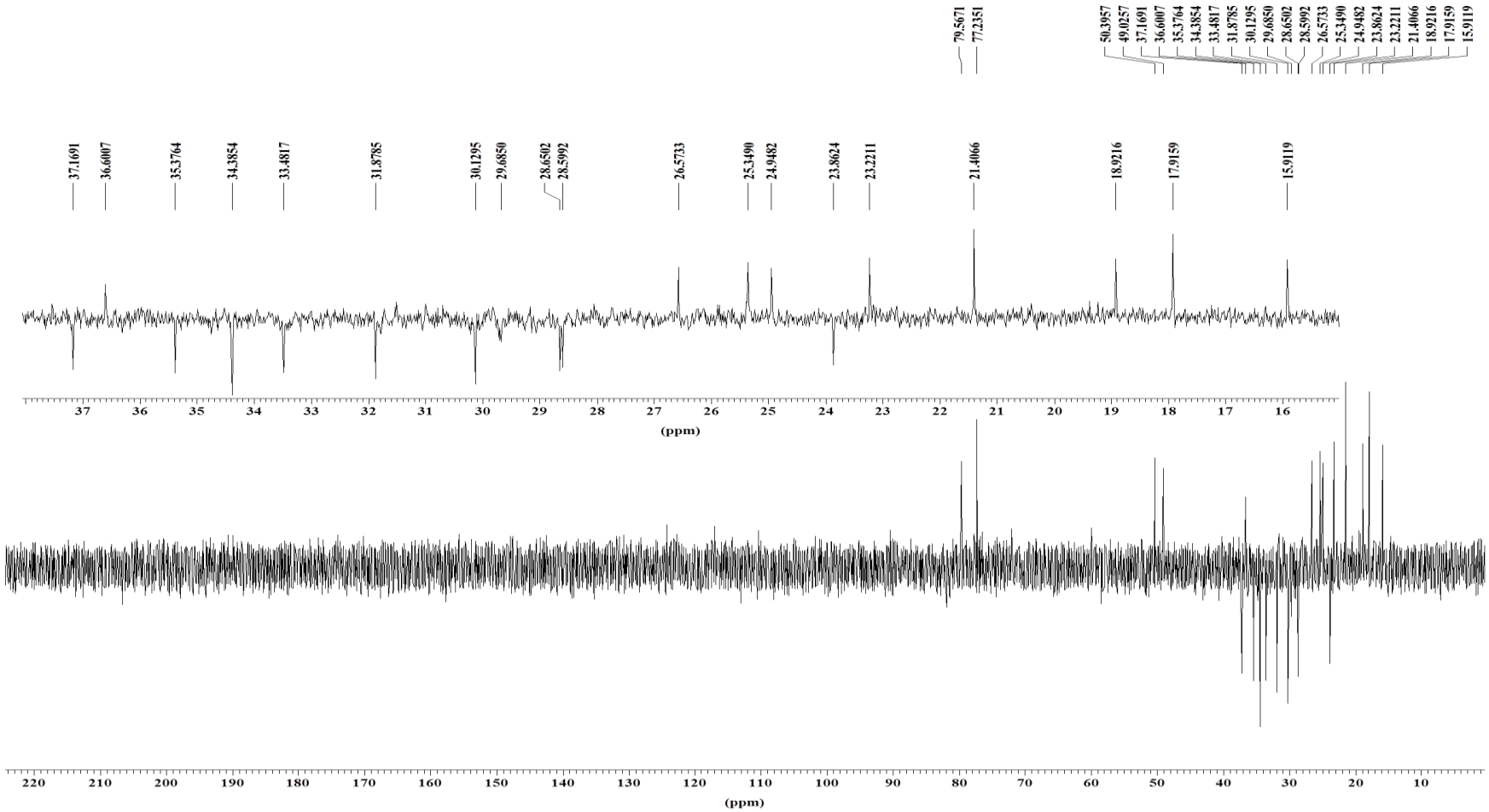
DEPT135 AMX500

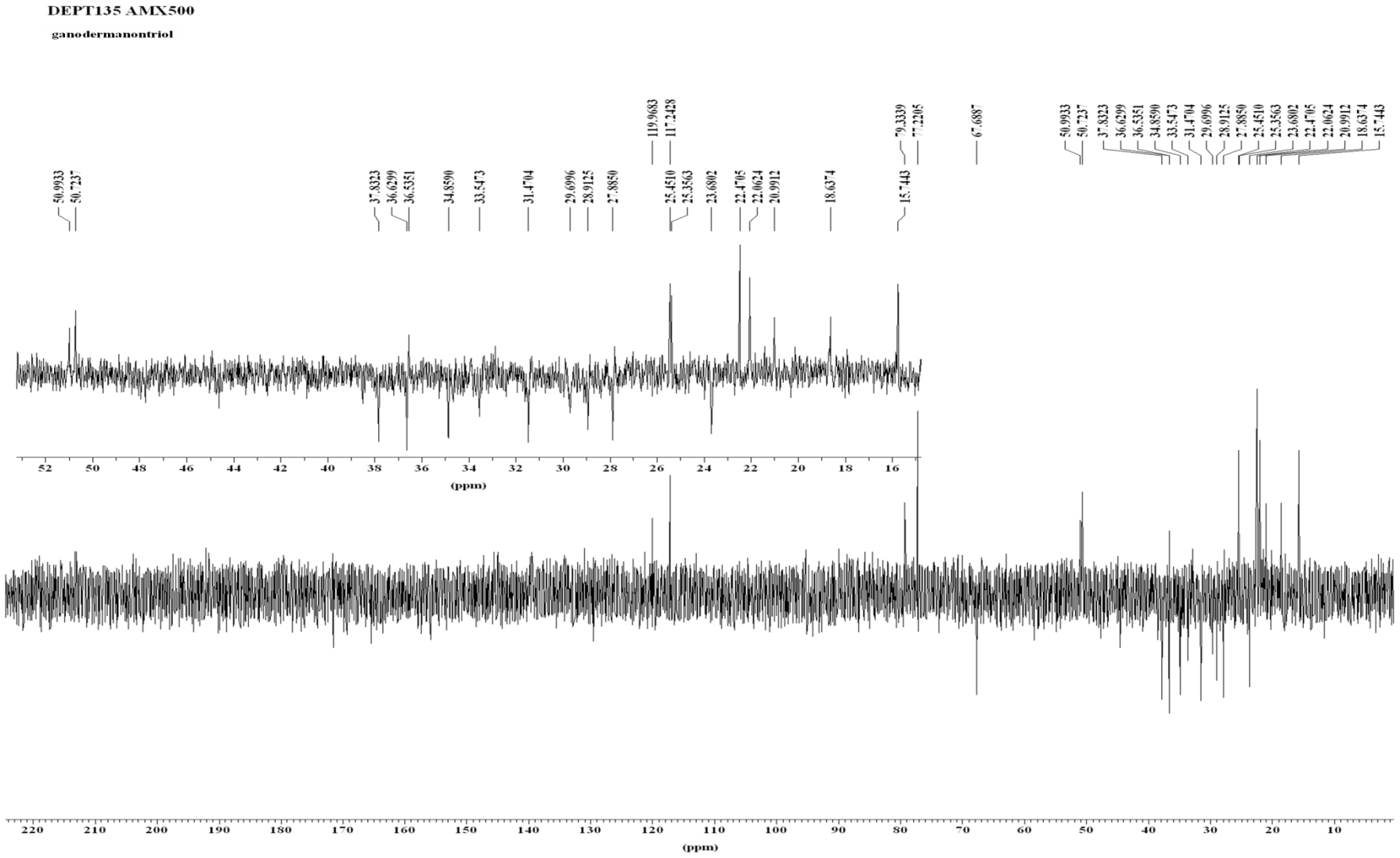
Ganolucidic acid E



DEPT135 AMX500

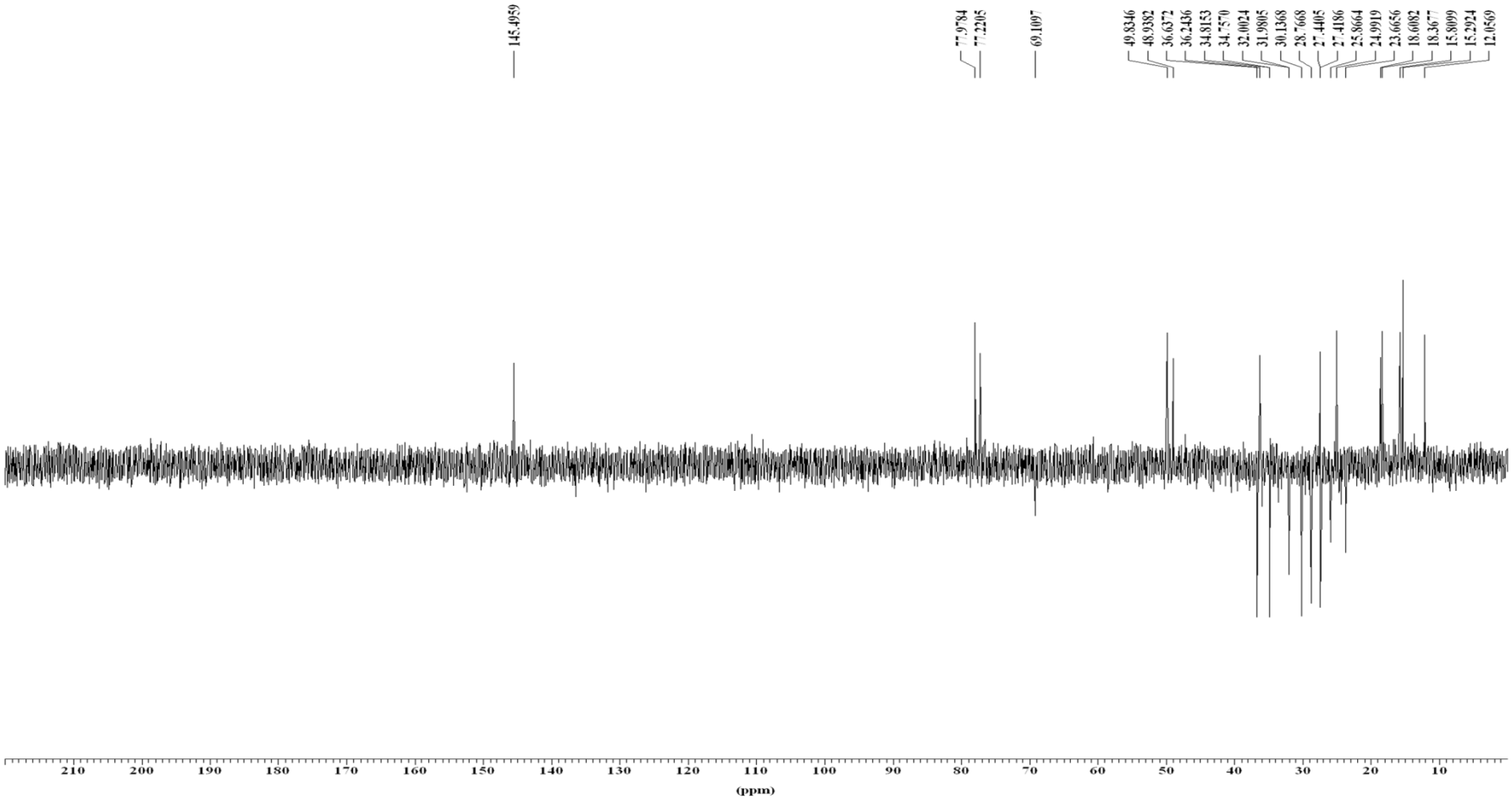
Lucidumol A




DEPT135 AMX500
ganodermanontriol
50.9933
50.7237
37.8323
36.6299
36.5351
34.8590
33.5473
31.4704
29.6996
28.9125
27.8850
119.9683
117.2428
25.4510
25.3563
23.6802
22.4705
22.0624
20.9912
18.6374
79.3339
77.2205
15.7443
67.6887
(ppm)
(ppm)

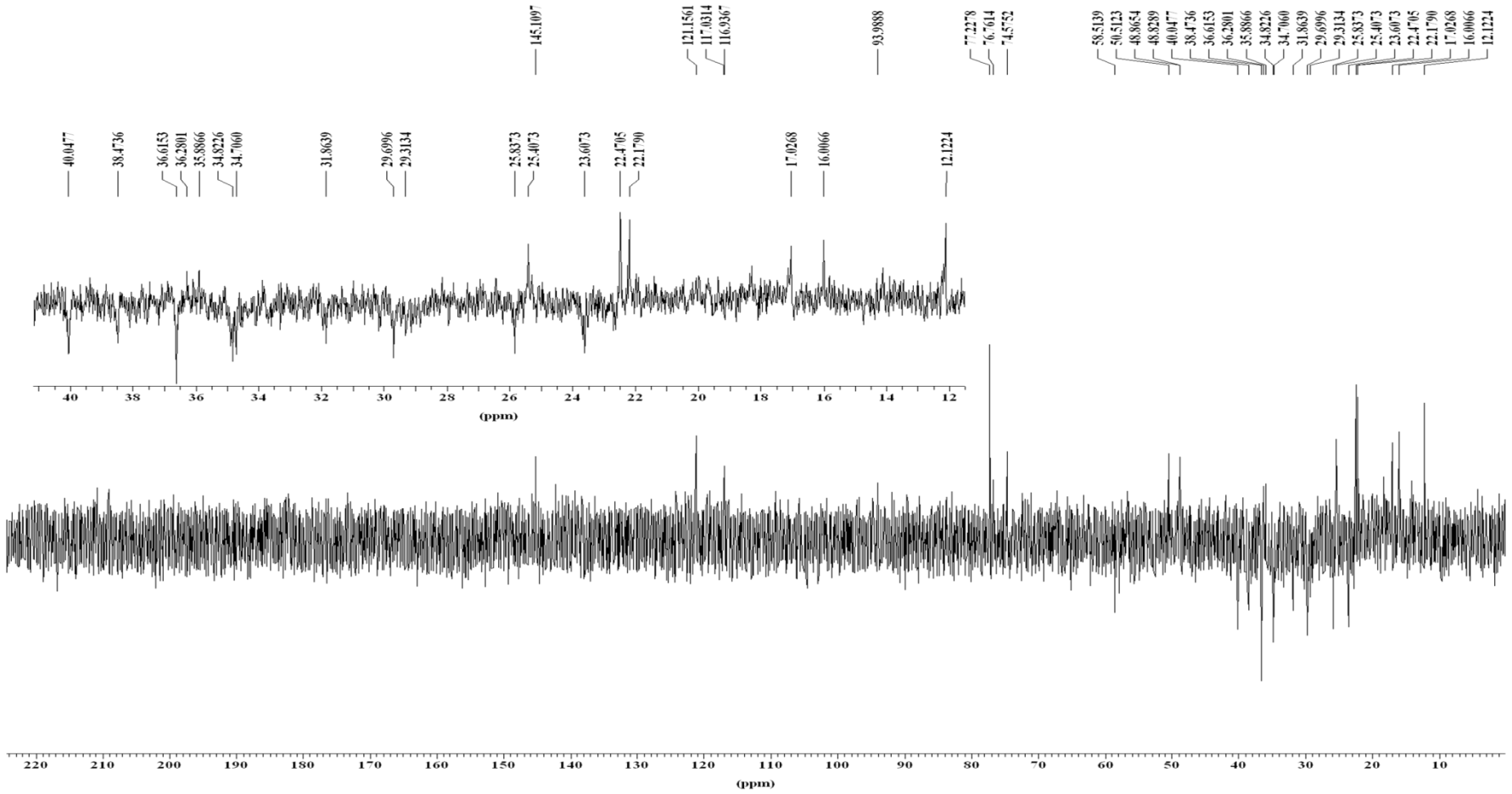
DEPT135 AMX500

7-oxo-ganoderic acid Z



DEPT135 AMX500

15-hydroxy-ganoderic acid S



DEPT135 AMX500

Ganoderic acid DM